THE EFFECT OF OPERATIVE INTERFERENCE WITH THE CERVICAL SYMPATHETIC NERVOUS SYSTEM UPON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

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Previous papers dealing with a transplantable neoplasm of the rabbit have emphasized the conception that variations in the character of the disease are largely due to variations in the mechanisms of reaction and resistance of the host and, furthermore, that this mechanism is an expression of animal metabolism or economy. It has been found, for instance, that the clinical course of the malignant disease may be correlated with the state of the glands of internal secretion as measured by the mass relationships of these organs (1). From our observations upon normal rabbits (2) as well as those infected with *Treponema pallidum* (3) it would appear that the alterations in mass relationship in the endocrine glands of tumor animals possess a functional significance and, furthermore, that as one of the factors in the constitutional organization of the animal, this system of organs occupies an important position in the mechanism of animal resistance. This view was submitted to direct investigation by means of various operative procedures with these organs and it was found that the growth and malignancy of the tumor could be affected in certain instances, notably by complete extirpation of the thyroid, a procedure markedly increasing the severity of the disease (4). It was felt, however, that the relative value of individual endocrine organs was of less importance than the integrity and balance of the system as a whole.

The possibility of a direct or indirect participation by the sympathetic nervous system in the reaction and resistance to the tumor has been considered with regard to the growth of the primary tumor and the subsequent course of the disease. In order to investigate it, various

parts of the sympathetic nervous system were removed before inoculation with the tumor. The cervical portion of the sympathetic system was selected because of its probable connection with the thyroid as well as its surgical accessibility.

Material and Methods.

Description of the Cervical Sympathetic Nervous System.—The cervical sympathetic nerve system in the rabbit comprises the upper two ganglia of the sympathetic chain on either side, together with the connecting trunk and rami of distribution. The superior ganglion lies at the base of the skull just medial to the internal carotid artery and vagus; the inferior ganglion is situated at the base of the neck directly above the origin of the subclavian artery; the trunk follows the course of the vagus through the neck. By analogy with human anatomy it is assumed that each ganglion is connected to the central nervous system by centripetal fibers (grey rami) and by centrifugal fibers (white rami). The grey rami join the corresponding cord segments in the case of each ganglion but the white rami for both ganglia enter the sympathetic trunk from the upper thoracic segments. Each ganglion, also, distributes fibers peripherally to blood vessels, glands, etc. It is not certain which of them, in the rabbit, is chiefly concerned in supplying the thyroid gland.

Operative Procedures.—Four types of operations were performed: (1) extirpation of both superior ganglia ("superior sympathectomy"), (2) extirpation of both inferior ganglia ("inferior sympathectomy"), (3) extirpation of both superior and both inferior ganglia and of both cervical sympathetic nerve trunks ("complete sympathectomy"), (4) division of the sympathetic trunk ("sympathotomy").

Ether anesthesia was used for each operation. No especial preparation was employed except the omission of the morning feeding on the day of operation. The hair over the ventral cervical region was clipped and shaved and the skin washed with 50 per cent alcohol. A midline incision was made, and by careful dissection in a bloodless field, the sympathetic trunk was isolated at a point just below the thyroid with retraction of the sternomastoid muscle medially. The trunk was then followed upwards or downwards to the ganglion to be removed.

Extirpation of the superior ganglion was easily accomplished because of ready approach and clear identification. It was avulsed, together with such of its fibers of distribution as came with it. Removal of the inferior ganglion, on the other hand, was much more difficult, principally because of its close relation to the subclavian artery and to a fine blood vessel plexus, and the approach to it was difficult also. Manipulation of the ganglion during a dissection was found to set up cardiodepressor reflexes occasionally resulting in sudden death. The operation of sympathotomy consisted in division of the sympathetic trunk in a midposition opposite the lower pole of the thyroid, by excising a small section between ligatures.

Our entire group of complete cervical sympathectomy operations numbers

thirty-two rabbits with an operative mortality of 18.8 per cent; however, among the last twenty animals there was but one death. Twenty-four rabbits have been operated upon for double inferior sympathectomy with an operative mortality of 16.7 per cent and but one death in the last fifteen animals. There were no deaths in the observation period between these operations and inoculation with the tumor, and we have had no fatalities with the superior cervical sympathectomy or sympathotomy operations.

The criteria of complete removal of the superior or inferior ganglia, or of the sympathetic nerve, were entirely objective, since the conditions of the work precluded the use of physiological tests. The dissections were so carried out that we could be reasonably certain during the operation of removing the entire ganglion, and its absence was verified by careful search at the postmortem examination of each rabbit. Extirpation of the cervical nerve or a portion of it was controlled by following the nerve to the superior cervical ganglion before removal or section of it.

Animals.—The animals employed were young adult or adult male rabbits selected and matched according to breed, age, and length of caging. They were separately caged and fed the ordinary diet of oats, hay, and cabbage used in these laboratories.

Tumor.—The tumor has been described in detail in previous papers (5, 6). It belongs to the class of epithelial tumors, is easily transplanted by intratesticular inoculation, and gives rise to a primary growth and metastatic growths in practically every tissue and organ of the body. The course of the disease in individual animals or in different series of animals is variable both as regards the actual outcome of the disease and as regards the course of the primary tumor and the incidence and distribution of metastases. Some rabbits die within 3 to 4 weeks after inoculation with extensive tumor involvement while others which may have shown a similar primary tumor growth ultimately recover and at postmortem examination show few or no foci of tumor. All gradations between these extremes are to be found.

Method of Inoculation.—At various intervals after operation inoculations were made with a salt solution suspension of an actively growing primary tumor; 0.3 cc. of the tissue emulsion was injected into one testicle. A suitable number of normal rabbits were inoculated at the same time to serve as controls.

Conduct of Experiments and Method of Analysis of Results.—Each rabbit was examined at frequent intervals with special reference to the following points: the rate, type of growth, and eventual fate of the primary tumor, the appearance of metastases in superficial parts of the body, and the general physical condition of the animal. Twice a week the animals were weighed and the primary tumor was measured. Rabbits which developed a paralysis of the legs or a physical deterioration with emaciation and weakness were killed with ether. The experiment was terminated 2 months after inoculation, at which time all surviving animals were killed. In each instance a complete postmortem examination was made with

special attention to the distribution, amount, and character of tumor tissue. In addition, all the principal organs were weighed.

The data obtained were used as a basis for estimating the character of the disease. The total probable mortality rate of a group includes those animals which died or were killed during the experimental period of 2 months and those which showed, at the end of this period, such metastatic growths as would probably have caused death at some future time as, for instance, tumors of both suprarenal glands. The incidence and distribution of secondary growths have been considered upon a relative and actual basis, the former including all animals of a group, while the latter takes into account only those in which metastases were found. The percentage estimations of the distribution of metastases have been calculated upon the basis of the number of foci theoretically possible as shown by the actual location of metastases in the first twenty generations of tumor animals (6).

The figures refer to the number of organs or tissues involved, not to the actual numbers of secondary growths, and, consequently, the expressions "foci of metastases," "distribution of metastases," or "metastatic rate" are used rather than "number of metastases." By contrast the figure for the number of clinical metastases detected during the life of the animal refers to the actual number of individual secondary tumors found.

There are certain obvious objections to the above method of estimation. For instance, such organs as the liver and kidneys may be markedly involved with numerous tumors or by only a few which destroy little of the organ. However, the general character of the disease process, whether of high, moderate, or low malignancy, is shown by a grouping of the percentage estimations of metastatic distribution according to the following four subdivisions (Text-figs. 2 and 3): (1) extensions and implantations including those to the retroperitoneal and mediastinal lymph nodes, (2) those to the lungs and pleura, liver, kidneys, and pancreas, (3) to the skin and subcutaneous tissue, superficial lymph nodes, muscles, bones and bone marrow, heart and pericardium, glands of internal secretion with the exception of the suprarenals, spleen, and the central nervous system, (4) to the suprarenals and eyes. This system of grouping was selected for the following reasons.

It has been found, from the study of several hundred rabbits inoculated with this tumor, that in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation there is usually a widespread distribution of metastases to the skin, the superficial lymph nodes, the muscles, the bones and bone marrow, the heart, the spleen, and the endocrine glands as well as to the parenchymatous viscera, the retroperitoneal and mediastinal tissues, and the serous membranes of the abdominal cavity. In instances of a somewhat less malignant disease, the most conspicuous and frequent secondary growths are found in the liver, kidneys, lungs, and pancreas. A level of still lower malignancy is chiefly characterized by the predominance of extensions to the retroperitoneal and mediastinal tissues and by implantations upon the omen-

tum, mesentery, and parietal peritoneum. If death occurs in animals so affected during the first 2 months after inoculation, the extensions and implantations referred to are found to be of an extreme grade or else, and more often, some organ such as the kidneys or hypophysis is also involved. Finally, in those animals in which the disease is very mild, metastases may be found only in such sites as the eyes or suprarenal glands which do not appear to share, to an equal extent, the resistance to tumor growth possessed or developed by other tissues and organs in the body. However, secondary growths in the eyes and suprarenals also occur in cases of extreme malignancy with a widespread distribution of metastases, so that with reference to the character of the disease it is necessary to separate the instances of a mild disease from those of a severe malignancy. In addition, it should be emphasized that metastases to the skin, muscles, bones, and endocrine gland group practically never occur in cases of low malignancy.

Number and Time of Experiments.—Our first experiments served to emphasize the necessity of a larger number of animals and the desirability of a uniform time interval between operation and inoculation. We have chosen as the basis for this report the third experiment in which such factors and that of the operative technique have been satisfactorily controlled. The series was inoculated on November 14, 1924, with the thirty-ninth generation of the tumor and consisted of 10 rabbits submitted to double superior sympathectomy (14 and 15 days between operation and inoculation), 9 with double inferior sympathectomy (16, 17, and 18 days between operation and inoculation), 10 with double complete sympathectomy (9, 11, 14, and 18 days between operation and inoculation), 9 with double sympathotomy (8 and 9 days between operation and inoculation), and 10 controls.

RESULTS.

The results of the experiment have been considered upon the basis of the general character of the disease developing in the various groups of animals rather than that of individual rabbits. The principal points are summarized in Tables I and II and Text-figs. 1 to 3. The disease of the normal or control rabbits will first be described as a means of comparison with that of the operated groups.

The primary tumor in the control rabbits grew in each instance; in two animals it was comparatively small, in two others it developed very rapidly and to extreme proportions during the first 3 weeks, while in the remainder it grew somewhat less rapidly but eventually attained a large size. The curves in Text-fig. 1 record the average calculated volume of the primary tumors of all the various groups of rabbits during the 1st month of the experiment, and it is seen that the volume in the control rabbits was somewhat greater than in the

TABLE I.

Analysis of Results.

Group No.	No. of rabbits.	Mortality.		Time of death.	Incidence of paralysis.	Clinical metastases.			Total foci of metastases.				Surviving rabbits.							Character of disease.		
															Negative or probable recovery.							
																Condition of primary tumor.		Metastatic foci.				
		Actual.	Probable total.			Animal incidence.	Total No.	No. per animal.	Animal incidence.	Total No.	Relative rate.	Actual rate.	No. of rabbits.	No. of probable deaths.	No. of rabbits.	Healed.	Necrotic.	No.	Relative animal incidence.	Severe.	Moderate.	Mild.
		per cent	*per cent*	*wks.*	*per cent*	*per cent*			*per cent*		*per cent*	*per cent*							*per cent*			
1	10	40	50	4 5 6 7 9	10	50	10	2.0	70	88	8.8	12.6	6	1	5	1	4	3	0.6	4	1	5
2	9	33	56	4 4 5 10 10	11	67	11	1.83	89	81	9.0	10.1	6	2	4	2	2	7	1.75	3	2	4

3	10	40	60	4 4 4.5 7 9 9	10	40	10	2.5	70	97	9.7	13.9	6	2	4	2	2	1	0.25	4	1	5
4	9	67	67	4 4 5 5.5 6 6	33	56	12	2.5	78	91	10.0	13.0	3	0	3	2	1	3	1.0	5	1	3
5	10	30	50	4.5 5 7.5 8.5 9.5	0	20	3	1.5	100	66	6.6	6.6	7	2	5	1	4	7	1.4	2	3	5

Group 1, superior; Group 2, inferior; Group 3, complete; Group 4, sympathotomy; Group 5, control.

operated animals with the exception of the sympathotomy group. Among the seven rabbits surviving at the conclusion of the experiment, the primary tumor was healed in one, largely necrotic in five, and mostly of living tissue in one instance. Three metastases were detected clinically in two rabbits. The actual mortality rate for this group was 30 per cent, the deaths occurring $4\frac{1}{2}$, 5, and $7\frac{1}{2}$ weeks after inoculation. However, it was felt, from postmortem examination at the end of the experiment, that two other animals would have eventually died from the effects of secondary tumor growths, thus making the total probable mortality 50 per cent. At autopsy one or more secondary tumors were present in each rabbit, an animal incidence of 100 per cent (Table I). The total number of metastatic foci in the group was 66, or a relative and an actual distribution rate of 6.6 per animal. A conception of the general distribution of these growths may be obtained by reference to Text-fig. 2 in which it is seen that the highest proportion of metastases occurred in the suprarenals and the eyes, while the smallest number were in the group comprising the skin, muscles, bones, and endocrine glands. As has been previously mentioned, metastases of the suprarenals and eyes usually occur either in cases of high malignancy with a widespread distribution of tumor or in instances of low malignancy in which such foci are frequently the only tumors found. There were six animals in the control group with metastases to these organs; in four the disease was mild while in two it was severe. Taking into consideration the mortality rate, the animal incidence of metastases, and the general distribution of the growths, the malignancy in the controls may be classed as of moderate or average severity.

In the four operated groups[1] the general severity of the disease or the level of malignancy was definitely higher than in the controls. The primary tumor always grew, but only in the sympathotomy group was its average volume comparable with that of the controls. In the case of the superior and complete sympathectomies the smaller size of the primary tumor was apparent 17 days after inoculation (Text-fig. 1), while by the 3rd week the tumors of the inferior group

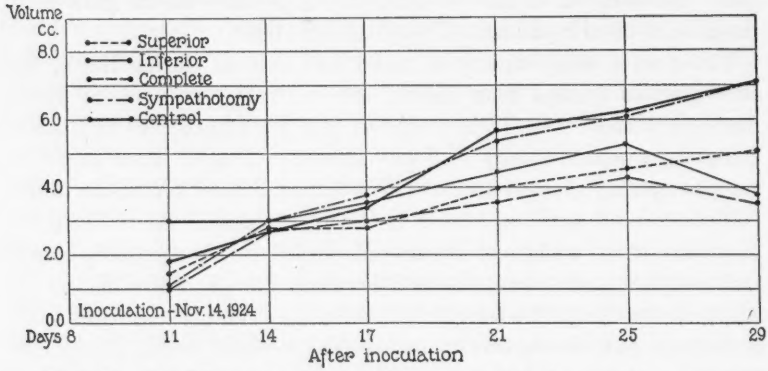
[1] The health and condition of the rabbits did not appear to be impaired as a result of the operations. The interval prior to inoculation was sufficient for recovery from any immediate ill effects.

were also definitely smaller than those of the controls and sympathotomy groups and continued to be so. At the conclusion of the experiment there were six surviving rabbits in the superior, inferior, and complete groups and three in the sympathotomy group. There was little difference in the character of the residual primary tumor among these groups except possibly in the case of the sympathotomy animals in which two were healed and one was practically entirely necrotic. There was one instance in each of the other three groups of a primary tumor which contained a considerable amount of living tissue. The relation of the character of the primary tumor growth to the general disease picture will be considered later.

There were more superficial metastases evident during life in all the operated groups than among the controls. Thus, after sympathotomy there were 12 in 5 rabbits (2.5 per animal), 10 in 4 after complete sympathectomy (2.5 per animal), 10 in 5 after superior sympathectomy (2.0 per animal), 11 in 6 after inferior sympathectomy (1.8 per animal), and 3 in 2 controls (1.5 per animal). As will be seen later, the most malignant disease developed in the sympathotomy and complete and superior sympathectomy groups, while the series with inferior sympathectomy fell between these and the controls. It so happens that the figures for metastases observed during life follow the same order.

The actual mortality rate (Table I) for the superior and complete sympathectomy groups, 40 per cent, was slightly higher than that for the controls, 30 per cent, while that for the inferior sympathectomies was the same, whereas the rate for the sympathotomies was more than double that of the controls, being 67 per cent. The greatest number of early deaths occurring, that is to say in the 4th and 5th weeks, occurred in the sympathotomy and complete sympathectomy groups. There was one instance in the complete, superior, and inferior sympathectomy groups and three in the sympathotomy group, of metastases to the spine causing paralysis of the hind quarters on which account the animals were killed. Among the controls there was one instance of deposits of tumor in the floor of the middle fossa of the skull beneath the dura. The total mortality rates of the several groups, which include those animals surviving throughout the experiment but which probably would have died from the effects of the tumor, approximate

each other more closely, but in the case of the sympathotomy rabbits the actual and total rates are the same, 67 per cent. There are slight differences, however, in the complete and superior sympathectomy groups which may be significant. Thus, there was an estimated mortality of six deaths in the complete sympathectomy group of which four actually occurred, and five in the superior sympathectomy groups with four actual deaths. On the other hand, among the five probable deaths in the inferior sympathectomy group and control animals, only three actually occurred.



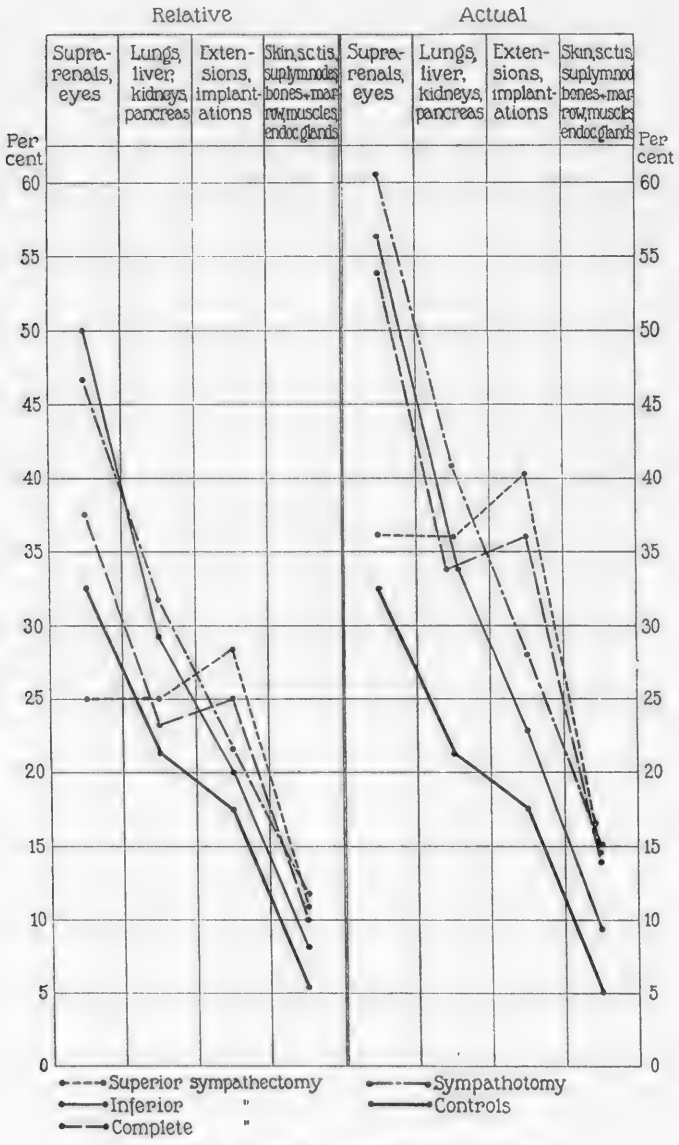
TEXT-FIG. 1. Effect of operative interference with the cervical sympathetic nervous system on the growth of the primary tumor.

With regard to the character of the disease as revealed by post-mortem examination, it is seen by reference to Table I that the highest animal incidence of metastases occurred in the controls. One or more foci of tumor tissue besides the primary tumor were found in each control animal, that is to say there was an animal incidence of 100 per cent, while the figures of the operated groups are as follows: inferior sympathectomy 89 per cent, sympathotomy 78 per cent, complete sympathectomy 70 per cent, superior sympathectomy 70 per cent. Here again, the inferior sympathectomy and control series approximate each other, while the other three operated groups fall closer together. The greatest distribution of metastases occurred in the complete sympathectomy and sympathotomy groups, the

numbers of metastatic foci being 97 and 91 respectively, while there were 88 in the superior sympathectomy, 81 in the inferior sympathectomy, with 66 in the control groups. In the sympathotomy series this distribution value may be considered as deceivingly low because of the number of what might be termed accidental deaths due to spinal metastases early in the course of the disease. Had these animals lived longer the metastatic rate would undoubtedly have been much higher and the actual amount of organ and tissue involvement considerably greater. The average number of metastatic foci per rabbit in which secondary growths occurred is as follows: complete 13.9, sympathotomy 13.0, superior 12.6, inferior 10.1, controls 6.6. The figures clearly show that upon a basis of distribution and growth of metastatic tumors the disease developing in the operated animals was considerably more malignant than among the controls, and, furthermore, that there were outspoken differences among the various operated groups which are of the same order as the mortality rate, the average number of clinical metastases per rabbit, and the total number of metastatic foci.

A more adequate conception of the general character or type of disease which developed in the several series of animals may be obtained by considering the distribution of metastatic tumors in various groups of organs according to the plan already described in the method of analysis of results.

The curves in Text-fig. 2 have been drawn upon the basis of organ and tissue grouping, the figures for the relative curves being based upon all the animals in a group, while those for the actual curves include only those animals in which metastases were found. The actual curves parallel the relative except at one point and, in general, merely emphasize the different types of disease which actually developed. At every point in both sets of curves, except in the relative curve in the case of the eyes and suprarenals of the superior sympathectomy group, the incidence and distribution of metastases are seen to be greater in the operated than in the control animals. The sympathotomy and inferior sympathectomy curves are similar in form, but the former is consistently higher and is considerably higher in the last column which records the involvement of the skin, muscles, bones,



TEXT-FIG. 2. **Distribution of metastatic foci.**

etc.[2] The curve representing metastases in the complete sympathectomy rabbits is almost the same as that of the inferior sympathectomy group in the first two columns (suprarenals and eyes; lungs, liver, kidneys, pancreas), but is much higher as regards extensions and implantations and definitely higher as regards skin, muscles, bones, and endocrine glands. The curve for the superior sympathectomy group resembles that of the complete sympathectomy animals, except in the case of suprarenal and eye metastases, in which it approaches the control curve.[3]

Upon the basis of these two sets of curves, one would conclude that the disease which developed in the four operated groups was on a much higher plane of malignancy than that of the control animals and, further, that the disease of greatest severity occurred in the sympathotomy and complete sympathectomy rabbits. In order to classify the inferior and superior sympathectomy series, however, an additional analysis is needed of the type of disease in which metastases occurred to the suprarenals and eyes. Relatively little importance has been attached to the incidence of extensions and implantations, but the fact that the figures for the superior sympathetic series are definitely greater than those for the inferior sympathetic animals in the groups of parenchymatous organs and the skin, muscles, bone, etc., indicates that the much greater value of the inferior sympathetic series for suprarenal and eye metastases is indicative of a mild or chronic disease rather than of greatly increased malignancy.

[2] Differences in the figures for the skin, muscle, bone, and endocrine gland group which appear comparatively small are actually of much greater significance than in the case of the other subdivisions because of the larger number of organs and tissues making up the group, the total figure for which is used in arriving at the percentage estimation values (6).

[3] While the level of malignancy of this superior sympathectomy group was much higher than that of the control animals, one of our preliminary experiments suggests that such may not always be the case. In this experiment there were only five superior sympathectomized rabbits, but the general character of the disease for the group was only slightly more severe than that of the controls. A possible explanation of this result may lie in the much longer interval between operation and inoculation—6 weeks—which may have been sufficient for such a readjustment of the animal organism that its resistance capacity reached the level for normal animals prevailing at that time.

The significance of the growth in the suprarenals and eyes is brought out in Table II which summarizes the principal points regarding the relation of these metastases to the total number of metastatic foci occurring in these rabbits as well as in all the animals of each group. The highest animal incidence and the largest number of suprarenal and eye tumors occurred in the inferior and sympathotomy series, but the inferior and control groups showed the greatest proportion of these growths as compared with the total number of metastatic foci of these rabbits (22.5 per cent), with the sympathotomy, complete,

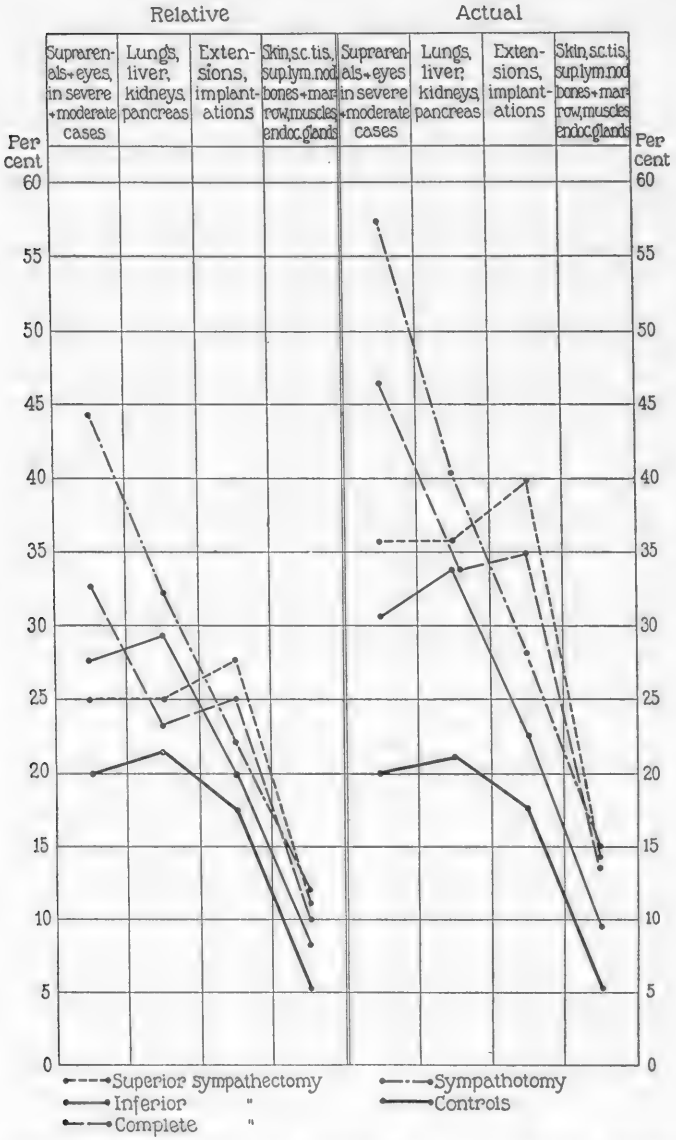
TABLE II.

Analysis of Metastases to Suprarenals and Eyes.

Group No.	No. of rabbits.	Proportion of rabbits with suprarenal and eye metastases to all rabbits with metastases.	Suprarenal and eye metastases.			Total No. of metastatic foci in entire group.	Proportion of suprarenal and eye metastases to metastatic foci of entire group.		Type of disease.			Proportion of mild types of disease.
			Total No.	Total No. of metastatic foci in these rabbits.	Proportion to total No.		Relative.	Actual.	Severe.	Moderate.	Mild.	
		per cent			*per cent*		*per cent*	*per cent*				*per cent*
1	4	59.1	10	68	14.7	88	25.0	35.7	3	1	0	0
2	7	87.5	18	80	22.5	81	50.0	56.3	3	1	3	42.8
3	6	85.7	15	96	15.6	97	37.5	53.6	4	1	1	16.6
4	7	100.0	17	91	18.8	91	47.2	60.7	5	1	1	14.2
5	6	60.0	13	58	22.5	66	32.5	32.5	2	0	4	66.7

Group 1, superior; Group 2, inferior; Group 3, complete; Group 4, sympathotomy; Group 5, control.

and superior groups following in the order named (18.8, 15.6, 14.7 per cent). Furthermore, when one classifies the type of disease which developed in the rabbits in which suprarenal and eye tumors were found, it is seen that the largest number of mild cases occurred in the control and inferior sympathectomy animals, 67 and 43 per cent respectively, while there were only 17 and 14 per cent of mild cases in the complete sympathectomy and sympathotomy groups, and none in the superior sympathectomy group. These points are graphically illustrated by a second set of curves from which the metastases to the suprarenals and eyes occurring in cases of low malignancy have



TEXT-FIG. 3. Distribution of metastatic foci omitting those to the suprarenals and eyes occurring in mild cases.

been omitted (Text-fig. 3), giving a more adequate conception of the precise character of the disease of the various groups. The curves representing the operated series are seen to be markedly higher than that of the controls. There is not a great deal of difference between the curves for the superior and complete sympathectomy groups, the former being slightly higher, except in the case of metastases to the eyes and suprarenals, but the disease of the inferior sympathectomy group is now clearly shown to be the least malignant of any occurring in the operated series. The order of malignancy of the several groups, as shown by these curves, is therefore as follows: sympathotomy, superior and complete sympathectomy, inferior sympathectomy, controls.

Finally, the condition of those rabbits surviving the experiment and not considered as probable ultimate deaths should be mentioned. The number of such animals is practically the same for all groups, four or five (Table I), except in the case of the sympathotomy series, in which there were three. A few foci of metastases were found at autopsy, from one to seven per group, and the relative animal incidence of these growths again brings out the resemblance of the inferior sympathectomy group to the controls (1.75 and 1.4 foci per animal), while the sympathotomy, the superior and complete sympathectomy groups are associated together on a plane of higher malignancy (1.0, 0.6, and 0.25 per animal).

DISCUSSION.

It is evident from this brief analysis that the various operations performed upon the cervical sympathetic ganglia and nerves brought about a condition of the rabbit host which permitted the development in the several groups of animals of a disease considerably more malignant than that prevailing among controls inoculated at the same time. Furthermore, there were differences in the malignancy level among the groups themselves. Upon a basis of actual and total probable mortality, the time of actual deaths, the number and animal incidence rate of clinical metastases, the total number of metastatic foci, and finally the general character of the disease as revealed by the distribution of these metastases, the order of malignancy in the operated groups beginning with that manifesting the most severe disease was as follows: sympathotomy, complete sympathectomy, superior sympathectomy, inferior sympathectomy, controls.

There were two points in which the disease of the controls might be considered as more malignant than in the operated animals. The average volume of the primary tumor was greater for the control group during the 1st month than that of any of the operated groups except the sympathotomy. It has been pointed out, however, that in a disease of a fairly high malignant level animals with the largest primary tumors are likely to show a less severe metastatic involvement than those with smaller primary growths (7). This generalization does not hold for a disease of a more severe character and consequently cannot be applied to the operated groups.

The rate for the animal incidence of metastases in the controls was 100 per cent, while that of the operated groups ranged from 70 to 89 per cent. In other words, tumor tissue in addition to the primary tumor was found in all the control animals, while two or three rabbits in each operated group had been able completely to prevent or suppress any metastatic growths. Although there was only a single focus of secondary tumor in four control rabbits, consisting of a cord nodule or a metastasis to one suprarenal gland, neither of which would have caused the death of the animal, still such a finding indicates that in certain rabbits or under certain conditions the surgical procedures employed did not invariably induce a state of decreased resistance to the tumor.

We have drawn attention to the fact that in any group of five or ten normal rabbits the proportion of those with high, low, and intermediate grades of resistance to this tumor, and to infections with *Treponema pallidum* as well, is roughly 1:1:3. The operative procedures employed in this experiment have not disturbed this ratio as regards the proportion of animals with high resistance, but apparently has done so in the case of the intermediate or low grades, since instances of moderate and severe malignancy of each group were considerably more numerous than in similar cases in the controls.

The interpretation of the facts that have been presented with regard to the general character of this malignant disease in rabbits after operative removal of various portions of the cervical sympathetic nerves can be only tentative, since our knowledge of the anatomy and physiology of these structures in the rabbit is slight. In particular there is no definite information available upon the connection between

the cervical sympathetic and the thyroid gland, though from what is known in the case of man and certain of the lower animals, it is probable that a relation of some sort exists. Our results furnish some evidence that both the superior and inferior ganglia are important structures in the mechanism of animal resistance to an induced malignant disease, the superior ganglia appearing to be the more essential in this relation. Whether it operates through the thyroid or by some other means is not certain, but the effects of complete thyroidectomy and of removal of the superior cervical ganglia, or of a portion of the sympathetic nerves, are, on the whole, strikingly similar in terms of general malignancy. There is in both cases a clear-cut increase in the severity of the disease. But, while removal of the thyroid practically always induces this effect, there are certain rabbits in which superior sympathectomy or sympathotomy did not. Furthermore, the rapid and very extensive growth of the primary tumors characteristic of completely thyroidectomized rabbits is not seen in superior sympathectomized animals nor is it equalled in the sympathotomized animals.

Removal of the inferior cervical ganglia resulted in a less malignant disease than that which developed in the superior and completely sympathectomized and the sympathotomized groups but one more severe than in the controls.

If the superior ganglia are the more important or essential elements in the cervical sympathetic system, then their removal alone or as a part of a complete cervical sympathectomy should bring about analogous effects. Such was apparently the case in this experiment. On the other hand, removal of a small portion of the cervical sympathetic nerve alone, without surgical interference of the superior or inferior ganglia, brought about a similar but even greater effect than removal of the superior ganglia or of the entire system. One might presume that extirpation of the inferior ganglia, in which the nerve is necessarily sectioned as in the sympathotomy operation, might bring about a similar effect. This did not occur, however.

The reasons for these various states of malignancy, or conversely, of animal resistance are not clear. The results of the experiment suggest several explanations as possible. The character of the malignant disease may be considered as a result of two major factors, (1) the

growth of the inoculated tumor cells and (2) the defensive forces which the animal host brings to play against these cells. There was a good initial growth of the primary tumor in every animal and we can assume that the operative procedures themselves did not interfere with or favor, to any material extent, the initial stage of the disease. On the other hand, since the eventual character of the disease as a whole was more malignant in the operated than in the normal animals, it is evident that the natural or acquired defensive forces of the host were in some way rendered less effective in the former.

When the superior ganglia were removed, either alone or as a part of a complete extirpation of the cervical system, or a medial portion of the nerves was removed, the mechanism of resistance was less efficient than when the inferior ganglia alone were extirpated. In both ganglion operations the nerve was necessarily cut, but one set of ganglia was not disturbed. It would seem, therefore, that these structures are associated with more than a single functional effect. There is probably a coordinating function of some kind between the two ganglia; yet, in addition, it would seem that both a favorable and a deleterious influence is associated with them. Thus, when the superior ganglia alone are extirpated a markedly unfavorable effect predominates; when the inferior ganglia alone are removed a much less deleterious effect is observed; whereas when both superior and inferior ganglia and the connecting nerves are extirpated the unfavorable effect is similar to that observed after removal of the superior ganglia. In other words, the absence of the inferiors does not increase or decrease the deleterious effect produced by the removal of the superior ganglia, so that it would seem that whatever unfavorable effect is produced by an inferior sympathectomy is mainly caused by an interruption of the path and hence of the coordinating function existing between the superior and inferior ganglia and that the additional removal of the inferior ganglia permits of the development of a favorable influence. Such an influence is not appreciable when the path between the ganglia is simply interrupted and the inferior as well as the superior ganglia are undisturbed. When this is the case, an outspoken deleterious effect upon resistance is to be seen, as witness, the severe character of the neoplastic disease. Under these conditions, the presence of either the superior or the inferior ganglia would appear to be a disadvantage.

If the effect of the various surgical procedures is obtained through the thyroid gland, the experiments would suggest that the absence of the superior cervical ganglia is associated with a condition of lowered or abolished thyroid function, as in the case of completely thyroidectomized rabbits in which there is a relatively ineffective resistance to the tumor. Furthermore, it would appear that since the absence of the inferior ganglia alone is associated with a less pronounced decrease in animal resistance the intact superior ganglia are still capable of exerting a favorable influence or of counteracting the unfavorable one brought about by the removal of the inferiors. However, since a distinct deleterious effect upon animal resistance followed an interruption of the direct nerve pathway between the undisturbed sets of ganglia, it is obvious that other factors are involved than are apparently associated with a simple extirpation of the ganglia.

Whatever may be the ultimate explanation of these apparently paradoxical results, it is clear, from this experiment, that removal of the cervical sympathetic system or certain of its component parts is, in the rabbit, followed in many instances by a less effective resistance to a transplantable neoplasm. In consequence, the character of the disease which develops in such animals is highly malignant.

SUMMARY AND CONCLUSION.

The effect of the removal of the complete cervical sympathetic nervous system, of both superior and of both inferior cervical ganglia, and of a small portion of the cervical sympathetic nerve in the rabbit was studied in relation to the character of a malignant disease induced by a transplantable neoplasm.

It was found that the general character of the disease which developed in the operated groups of animals was more severe than that of a similar sized control group. Comparisons of the mortality rate in the several groups, of the animal incidence of metastases, the number of metastatic foci, and the distribution of these secondary growths all showed this to be the case. There appeared, furthermore, to be differences in malignancy among the operated groups themselves. The most severe disease occurred in the group in which a portion of the sympathetic nerve only was removed (sympathotomy); that in the

complete sympathectomy and superior sympathectomy groups was slightly less malignant; and that in the inferior group was much less so.

These results have been interpreted as due to a less effective animal resistance, the mechanism of which has been interfered with in some way by the interference with the sympathetic nerves. The reasons for the difference in malignancy exhibited by the several operated groups are undetermined. A tentative explanation is suggested upon the basis of coordinating, favorable or deleterious functions subserved by the cervical sympathetic nervous system.

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COMPARATIVE STUDIES OF THE ACTION ON THE PNEUMOCOCCUS OF BILE ACIDS AND UNSATURATED FATTY ACIDS, FOUND IN BILE IN THE FORM OF SOAPS.

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Up to the present time, in studying the action of bile on the pneumococcus, only the bile acids and whole bile have been taken into consideration; the presence in bile of higher unsaturated fatty acids which are there in the form of soaps and in such a concentration that their part in the action of bile on the pneumococcus cannot be disregarded, has been entirely overlooked. Moreover, only the phenomenon of the solubility of pneumococci in bile or bile acids has been attentively considered, no attempt being made to determine which bile substance is most effective in its antiseptic action on the pneumococcus or other bacteria.

This question is not without significance when one takes into account that, before their elimination into the gall bladder, bile substances are present in the liver tissues and blood where they may play a very important rôle in the infectious diseases. With that in mind, I have studied the action of the conjugated bile acids, cholic acids, and unsaturated higher fatty acids found in bile in the form of soaps. As no pure commercial products were available, the substances used were prepared in this laboratory.

Preparation of Crystallized Bile.[1]

The fresh ox bile mixed with twice its volume of 95 per cent alcohol was allowed to stand in the cold room overnight. The precipitated mucins were separated by decantation and filtration and were washed in the filter three times with 95 per

[1] The preparation of the bile acids was carried out by methods on which those of Hammarsten (1) and Schryver (14) are based.

cent alcohol. The washings were added to the filtrate. The alcoholic bile was concentrated by distillation under diminished pressure, mixed with animal charcoal, and further evaporated on the water bath with the aid of an electric fan. The residue was dried *in vacuo* over sulfuric acid and extracted with hot absolute alcohol. The alcoholic filtrate was precipitated with ether. The precipitate was dissolved in water and further decolorized by animal charcoal and again extracted with absolute alcohol. To the alcoholic filtrate, anhydrous ether was added until permanent cloudiness developed and the fluid was allowed to stand overnight in a cold room. The white precipitate of bile salts was separated by decantation, washed with ether, and used for experiments as "crystallized bile," which is a mixture of conjugated bile acids and may contain a small amount of salts of fatty acids.

Preparation of Glycocholic Acid.

To the "crystallized bile," dissolved in water to the concentration of 2 per cent, a solution of neutral lead acetate was added while stirring. The precipitated lead glycocholate was filtered off and dried in a desiccator over calcium chloride *in vacuo*. The dry substance was suspended in hot alcohol and the lead was removed by sulfurated hydrogen. From the concentrated alcoholic filtrate, the free glycocholic acid was precipitated by ether and recrystallized from boiling water on cooling.

Preparation of Taurocholic Acid.

Taurocholic acid was prepared from ox bile freed from mucins by alcohol and from glycocholic acid by ferric chloride. After the evaporation of the alcohol, the acid was precipitated by basic lead acetate and purified in the same manner as glycocholic acid but recrystallized from alcohol by the addition of ether containing a little water.

Preparation of Cholic Acids.

A concentrated solution of sodium hydroxide was added to the fresh ox bile to make in it an 8 per cent solution of NaOH. The fluid, with the admixture of a little sand, was boiled over a gas flame through an asbestos wire gauze. While heating under a reflux condenser, the flame was regulated to avoid foaming into the condenser. The heating was interrupted at night. After 30 hours of boiling, the warm fluid was diluted with twice its volume of water and while still warm a dilute hydrochloric acid was slowly added while stirring the fluid with a glass rod. A rich, greyish white precipitate was formed and a viscid dark yellow mass of cholic acids in the form of small pasty granules which were gathered up on the end of the rod and transferred into a beaker; by that means, the larger part of the cholic acids was separated mechanically. The rest of the cholic acid was recovered by the extraction of the precipitate with hot acetone and added to the mass already isolated. After evaporation of the acetone, the dry residue was powdered and washed with cold water, redissolved in hot acetone, and filtered hot.

After evaporation of the acetone, the residue was dissolved in diluted ammonia and decolorized by animal charcoal. The acids were then precipitated from the filtered solution by the addition of diluted hydrochloric acid. The precipitate was washed with water and dried *in vacuo* over calcium chloride and soda-lime, dissolved in acetone, and filtered while hot. On cooling the concentrated solution, crystals of the cholic acids were precipitated and were then washed in a filter with a small amount of cold acetone. The filtrate and washings together yielded, after concentration, a further quantity of crude cholic acids which contained a mixture of cholic, choleic, and desoxycholic acids.

Separation of cholic, choleic, and desoxycholic acids was based on the difference in the solubility of their magnesium and barium salts. For this purpose, to the 1 per cent watery solution of sodium salts of crude cholic acids was added one-tenth its volume of 20 per cent solution of magnesium chloride and the mixture was heated for an hour on the water bath; a white precipitate was obtained which consisted of magnesium salts of choleic and desoxycholic acid while the magnesium salt of cholic acid remained in solution and the cholic acid was recovered by acidification of the filtrate. The choleic and desoxycholic acids were obtained by the decomposition of their magnesium salts suspended in water after acidification with hydrochloric acid. They were then dissolved in diluted ammonia, and 1 per cent solution of barium chloride was added. A precipitate was formed, and separated by filtration. This precipitate, washed with water, yielded, after acidification with diluted hydrochloric acid, the choleic acid, while from the filtrate after acidification, the desoxycholic acid was obtained.

The acids thus separated were further purified by conversion into their magnesium and barium salts and by recrystallization from hot acetone.

The Isolation of Fatty Acids from Ox Bile.

An amount of 4300 cc. of bile was collected from fresh gall bladders with all precautions to avoid contamination by fats from other tissues. The specific gravity of the fluid was determined as 1.03. The mucins, which are troublesome, were precipitated by alcohol, removed by filtration, and extracted repeatedly with 95 per cent alcohol. The alcoholic extracts were added to the filtered bile. After evaporation of the larger part of the alcohol by distillation under diminished pressure, 10 cc. per liter of 30 per cent potassium hydroxide were added and the fluid was extracted five times with petroleum ether until it was colorless and no residue remained after the evaporation of a sample.

By this means, the neutral fats and lipoids were removed from the bile in an amount of 22.65 gm. After that, the alkaline bile was acidified with diluted hydrochloric acid and again extracted several times with petroleum ether which now separated from the bile the fatty acids originally present in it in the form of soaps. The ethereal solution, washed with water and dried *in vacuo*, yielded a substance the larger part of which was crystallized in white needles with an admixture of an oily yellow fluid. The amount of this substance was 14.6 gm.,

and its iodine value of 50, determined by Wys' (16) method, proved that it contained a large amount of unsaturated compounds.

The separation of unsaturated fatty acids was accomplished by Varrentrapp's method based on the difference in the solubility of their lead salts. For this purpose, the fatty acids were dissolved in absolute alcohol; to this hot solution was added lead acetate dissolved in hot alcohol. After cooling, a white precipitate was formed which consisted of the lead salts of saturated fatty acids while those of unsaturated fatty acids remained in solution. The precipitate was washed with ether and the washings united with the filtrate which was concentrated and treated with a diluted hydrochloric acid and extracted with ether. The ethereal solution, washed with water, gave, after evaporation, a brownish yellow oily mixture in an amount corresponding to 0.17 per cent of whole bile and consisting of unsaturated higher fatty acids; the iodine value of this substance was 160, which indicates the presence not only of oleic but also of higher unsaturated fatty acids.

The oleic acid was separated from other more unsaturated fatty acids by means of barium salts. For this purpose, the mixture of unsaturated fatty acids was dissolved in a diluted ammonia and a solution of barium chloride was added while stirring. This precipitate was separated by filtration, washed with water, and partially dried between blotting paper and after that *in vacuo* over calcium chloride. The dry barium salts were dissolved in hot benzene containing 5 per cent of 95 per cent alcohol and allowed to stand overnight in the cold room. The white precipitate of barium oleate was formed; this was removed by centrifugalization, washed with alcoholic benzene, redissolved in hot alcoholic benzene, and decomposed by diluted hydrochloric acid. One obtained from this fraction an oily liquid, solidifying at 0°C. and melting at about 10°C.; its iodine value, determined by Wys' method, was 80, which approximates the theoretical value of oleic acid (90). Its amount corresponded to 0.07 per cent of whole bile. From the barium salts, which remained in solution in cold alcoholic benzene, one obtained after decomposition, purification, and drying *in vacuo*, a dark yellow oily liquid in an amount corresponding to 0.1 per cent of whole bile. Its iodine value was 174, which approximates that of linolenic acid (181). The fluid did not solidify at −6°C.

The fatty acids were further identified by the bromide test. While the fraction with an iodine value of 80 gave no solid bromides, the fraction with an iodine value of 178, brominated in ethereal solution acidified by acetic acid, gave crystallized bromides insoluble in petroleum ether and in cold benzene but almost entirely, although not completely, soluble in hot benzene. This reaction indicates that among the unsaturated fatty acids obtained from bile, there were present not only the acids with one and two, but also with more double bonds.

Preparation of Sodium Salts.

All the above mentioned bile acids and unsaturated fatty acids have been converted into sodium salts to bring out the action of anions. For this purpose, a

substance was dissolved in absolute alcohol, 3 drops of 1 per cent alcoholic solution of phenolphthalein were added, and a fresh alcoholic solution of sodium hydroxide was added drop by drop until a faint alkaline reaction appeared. The precipitated salts were washed with alcohol and ether. The further yield of salts was recovered from the concentrated and finally evaporated filtrate.

Comparison of the Action on the Pneumococcus of the Bile Acids and Unsaturated Fatty Acids.

In studying the action of the above mentioned substances on the pneumococcus, the highest (*a*) inhibitory and (*b*) bactericidal dilutions were determined.

The preliminary experiments showed the highest antiseptic dilution to be as follows: conjugated bile salts about 1:300; sodium choleate 1:1000; salts of unsaturated fatty acids 1:30,000. For the more precise experiments, the solutions of bile acid salts prepared were 5, 2.5, 1, 0.5, 0.166 per cent, and lower. These solutions were sterilized in the autoclave for 15 minutes at 120°C. under 15 pounds pressure.

1 cc. of a solution was added with all antiseptic precautions to the test-tubes containing 4 cc. of beef infusion broth to obtain the series of dilutions: 1:100, 1:200, 1:500, 1:1000, 1:2000, and so on up to the dilution 1:5000. To the control test-tubes, containing also 4 cc. of broth, was added 1 cc. of distilled water. The soap solutions were prepared in a parallel manner starting from 0.1 per cent solution up to 0.005 per cent to obtain the dilutions 1:5000, 1:10,000, 1:20,000, 1:30, 000, and so on up to 1:100,000.

For the comparative examination of different types and strains, one solution of salt (1 per cent solution of bile acids and 0.1 per cent solution of soaps) was made and it was added in corresponding amounts to a series of flasks each containing 200 cc. of broth to get the desired dilutions. Medium thus prepared was dispensed aseptically in the test-tubes, sterilized in the Arnold autoclave at 100°C. for 20 minutes, and incubated at 37°C. for 3 days to insure sterility and after that inoculated with pneumococci.

The medium used for all the experiments was beef infusion broth prepared as follows:

Beef chopped	1 lb.
Distilled water	1 kg.
Peptone	1.0 per cent.
Sodium chloride	0.5 " "
(pH 7.6)	

The reaction of the medium after the addition of the solution was only very slightly changed (pH 7.5).

(*a*) *Inhibitory Action.*—To the test-tubes containing corresponding dilutions of bile substances in broth, prepared as previously described, 0.2 cc. of an 18 hour pneumococcus culture was added and the tubes were incubated at 35°C. for 4 days. From those in which the broth remained entirely clear, or clouded for only a short time, transfers were made at different time intervals in an amount of one loopful in one, and of 0.2 cc. in the other series of test-tubes containing pure broth; the re-inoculated test-tubes were incubated under the same conditions and observed for 4 days. The results are given in Table I.

(*b*) *Bactericidal Action.*—The test-tubes containing exactly 4 cc. of broth were inoculated with 0.2 cc. of an 18 hour pneumococcus culture and incubated for 4 hours at 35°C. In this time, the growth was sufficient to make the broth distinctly cloudy. After that, 1 cc. of a corresponding solution of bile substances was added to get the same series of dilutions as in the first experiment. The test-tubes were replaced in the incubator and from time to time transplants were made from the tubes in which broth became clear.

TABLE I.

Action of Bile Substances on Pneumococcus Growth.

Substance.	Dilutions in which		
	Growth was entirely inhibited and the bacteria killed in less than 24 hrs.	Growth inhibited and bacteria alive after 24 hrs.	Growth partially inhibited.
Whole ox bile sterilized at 100°C. for 10 min..	1:10	1:50	1:100
Crystallized bile	1:300	1:500	1:1000
Sodium glycocholate	1:300	1:500	1:1000
" taurocholate	1:500	1:1000	1:2000
" cholate	1:1000	1:2000	1:3000
" choleate	1:1000	1:2000	1:3000
" desoxycholate	1:1000	1:2000	1:4000
" salt of unsaturated fatty acid with iodine value 80	1:30,000	1:40,000	1:70,000
Sodium salt of unsaturated fatty acid with iodine value 174	1:50,000	1:70,000	1:100,000

The bactericidal dilutions were found to be a little higher than the figures in the first column of Table I. The bactericidal action of higher unsaturated fatty acids and bile acids depends on the same phenomenon; that is, on the dissolving of pneumococci (compare Tables II and III) although the first are about 100 times stronger than the second (compare Table I). The reaction of the several types and

TABLE II.

Action of Sodium Taurocholate on Pneumococcus Growth.

(*a*) To test-tubes containing 4 cc. broth was added 1 cc. of corresponding solution; after that, 0.2 cc. of pneumococcus culture was inoculated.

Time.	Dilutions.							
	1:200	1:500	1:1000	1:2000	1:3000	1:4000	1:5000	Control.
hrs.								
4	−	−	−	−	±	+	+	+
10	−	−	−	±	+	++	++	++
24	−	−	−	−	±	+	++	++
48	−	−	−	−	−	±	±	+
in transplants made after								
hrs.								
4	−	+	+	+				
10	−	−	+	+				
24	−	−	+	+				
48	−	−	−	−	−	+	+	+

(*b*) Test-tubes containing 4 cc. broth were inoculated with 0.2 cc. pneumococcus culture and incubated at 35°C. for 4 hours; after that, 1 cc. of corresponding solution was added.

hrs.								
1	±	+	++	++	++	++	++	++
4	−	−	−	±	+	++	++	++
10	−	−	−	−	±	+	++	++
24	−	−	−	−	−	±	+	++
48	−	−	−	−	−	−	±	+
in transplants made after								
hrs.								
4	−	−	+	+	+	+	+	+
10	−	−	−	+	+	+	+	+
24	−	−	−	−	+	+	+	+
48	−	−	−	−	−	−	+	+

− = broth clear.
± = broth slightly cloudy from growth of the pneumococcus.
+ = broth distinctly cloudy from growth of the pneumococcus.
++ = broth very cloudy from growth of the pneumococcus.

TABLE III.

Action of Sodium Oleate on Pneumococcus Growth.

(*a*) To test-tubes containing 4 cc. broth was added 1 cc. of corresponding solution; after that, 0.2 cc. of pneumococcus culture was inoculated.

Time.	Dilutions.											
	1:5000	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:90,000	1:100,000	Control.
hrs.												
4	−	−	−	−	−	−	±	±	+	+	+	+
8	−	−	−	−	±	±	±	+	++	++	++	++
18	−	−	−	−	−	±	±	±	+	++	++	++
24	−	−	−	−	−	−	±	±	±	++	++	++
in transplants made after												
hrs.												
4	−	−	+	+	+	+	+					
18	−	−	−	−	+	+	+					
24	−	−	−	−	−	+	+	+	+	+	+	+

(*b*) To 4 cc. broth culture, incubated at 35°C. for 4 hours, was added 1 cc. of corresponding solution and further incubated.

min.												
30	−	+	+	++	++	++	++	++	++	++	++	++
hrs.												
1	−	±	+	+	++	++	++	++	++	++	++	++
2	−	−	−	+	+	+	++	++	++	++	++	++
4	−	−	−	−	−	±	+	++	++	++	++	++
10	−	−	−	−	−	−	−	+	++	++	++	++
12	−	−	−	−	−	−	−	−	+	++	++	++
24	−	−	−	−	−	−	−	−	±	+	++	++
in transplants made after												
hrs.												
1	−	+	+	+								
2	−	−	−	+	+	+						
4	−	−	−	−	−	+						
10	−	−	−	−	−	−	−	+				
24	−	−	−	−	−	−	−	−	+	+	+	+

− = broth clear.
± = broth slightly cloudy from growth of the pneumococcus.
+ = broth distinctly cloudy from growth of the pneumococcus.
++ = broth very cloudy from growth of the pneumococcus.

strains of the pneumococcus is different. Some strains are more or less sensitive to the action of the bile substances examined, and even in the culture of the same strain some cells are more resistant than others.

DISCUSSION.

In 1900, Neufeld (11) first drew attention to the solubility of the pneumococcus cell in bile. He attributed this action exclusively to the bile acids. In the report of his research on the hemolytic action of oleic acid (12) (1908) he says:[2] As regards their effect on the pneumococcus, the bile acid salts occupy an entirely isolated position. In particular it should be mentioned that neither soap nor concentrated solutions of potassium hydroxide, nor "sapotoxin" dissolve pneumococci. That this assertion is not entirely justified has been shown by Lamar (5) (1911) who, in his studies on the influence of unsaturated fatty acids on the solubility of the pneumococcus by immune serum, gave figures which are not far from those obtained in my experiments. The amount of higher unsaturated fatty acids I isolated was 0.17 per cent (crude substance) of whole bile; that is, they are present there in a dilution of about 1:600 in which they kill the pneumococcus in a few minutes, while the conjugated bile acids were found in ox bile by Hammarsten (1) and many others in a concentration of from 0.5 to 1 per cent; that is, they are present in bile in a dilution of from 1:200 to 1:100, in which they kill pneumococci after about 10 hours or more. In this case, the presence of higher unsaturated fatty acids in bile is more important than that of bile acids.

The presence of soaps of unsaturated fatty acids in bile should be taken into consideration not only by the bacteriologist but also by the physiologist and biochemist as these substances seem to be in a genetical relation to cholesterol and bile acids (Lifschütz (8)), and they may play a very important rôle in fat metabolism (Leathes (7)) and in jaundice.

In this connection there are only two suggestive articles; one by Trifanovsky (15), the other by Lassar-Cohn (6). The former (1874) found in the alcoholic extract from human bile, besides the bile acids, also the saturated higher fatty

[2] Neufeld and Händel (12), p. 581.

acids with an admixture of an oily substance which he believed to be oleic acid but did not study further. Lassar-Cohn (6) (1893) in the preparation of cholic acid found an oily substance the lead salt of which was soluble in ether, a fact which caused him to suggest that it was oleic acid; but he also did not undertake any further examination. By the method he used (boiling of bile with 6 per cent sodium hydroxide for 24 hours—Mylius' (10) method) not only the conjugated bile acids can be dialyzed but also the neutral fats and phospholipins. One cannot be sure then whether the oleic acid he obtained had its origin in the neutral fats or in the soaps of bile. The remarks found in different handbooks should therefore be corrected (Oppenheimer (13); Hoppe-Seyler (3)) that Lassar-Cohn (6) has detected (1893) the salts of oleic acid in bile. The same objection can be made to Mathews (9) who claims in his handbook that Hammarsten (2) has detected the soap of oleic acid in bile, as in the cited papers one finds that Hammarsten distinctly states that he obtained it by the hydrolysis of neutral fats of bile. No other significant articles or observations concerning oleic acid or the more unsaturated fatty acids in bile were found in the literature.

Kauftheil and Neubauer (4) (1924) have recorded a little lower bactericidal limit of desoxycholic acid (1:200) for the pneumococcus than in my investigations, which is possibly due to the fact that they used a different culture medium.

SUMMARY.

Ox bile contains the soaps of unsaturated higher fatty acids not only with one, but also with two and more than two double bonds, in a dilution of about 1:600. These substances, when isolated from bile, exert an antiseptic action on the pneumococcus in a dilution of approximately 1:50,000 and kill the organism in a dilution of 1:5000 in approximately 1 hour. As their action is, then, about 100 times stronger than that of the conjugated bile acids, their presence in bile should be considered, whereas hitherto it has been entirely overlooked not only by bacteriologists but by physiologists as well. Since the soaps of unsaturated fatty acids do dissolve the pneumococcus cell in broth culture, Neufeld's (12) conclusion is no longer valid.

In conclusion, I wish to express my appreciation and thanks to Dr. Augustus B. Wadsworth, Director of the Division of Laboratories and Research of the New York State Department of Health, Albany, whose kindness has made it possible for me to carry out these investigations.

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LOCAL IMMUNIZATION OF RABBITS TO CUTANEOUS INFECTION WITH STAPHYLOCOCCUS AUREUS.

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In a series of papers published from 1922 to 1924 Besredka (1) and his collaborators have described methods of intra- and transcutaneous vaccination by which they have claimed successful immunization to cutaneous infection with streptococci and staphylococci; and they have reported astonishingly favorable results from clinical applications of their methods. The most successful of these results were obtained with filtrates of broth cultures injected intracutaneously or merely applied to large areas of freshly shaven skin in the form of poultices. These filtrates were prepared by incubating broth cultures for 10 days, filtering, reinoculating, incubating for another 10 days, then refiltering. Such filtrates, they report, would no longer support the growth of the specific organism, although other organisms, such as typhoid bacilli, grew well in them. They believe that a growth-inhibiting factor, specific in its action, develops in such cultures and that this substance is capable of inducing active immunity within a period of 24 hours, if infiltrated into or poulticed upon the skin, but is quite ineffective if injected subcutaneously, intravenously, or intraperitoneally.

Rivalier (2), working with streptococci in rabbits, found that filtrates prepared by Besredka's method conferred a strictly local immunity to the area of skin treated, but that other areas remained normally susceptible. In spite of repeated cutaneous infections with virulent organisms, his animals never developed an immunity to infection in untreated areas of skin. Rivers (3), in work published since our own investigation was undertaken, found that repeated cutaneous infection with strains of streptococci isolated from cases of

human erysipelas led to a general immunity of the skin, and that the infiltration of areas of skin with sterile infusion broth produced a strictly local immunity of the area treated within 24 hours.

Production of Filtrates.

Bacterial filtrates were prepared in the manner described by Besredka by inoculating flasks of extract or infusion broth with staphylococci from human sources, incubating 10 days, filtering, reinoculating, and refiltering after a second period of incubation. In many instances this process was repeated up to four reinoculations but the filtrate never lost the power of supporting the growth of the strain of organisms used. Similar results were obtained in preparing filtrates of streptococci, although in this case it was necessary to correct the hydrogen ion concentration before reinoculating in order to assure growth. Filtrates of cultures varying in age from 4 days to 10 weeks were used without variation in the results obtained.

Animal Experiments.

Preliminary experiments quickly showed that no general protection was conferred upon the animals either by the intracutaneous inoculation of the filtrates or by the poulticing of large areas from 10 to 15 cm. in diameter with dressings soaked in the filtrates. In most of our experiments, therefore, test and control areas were selected in symmetrical locations on opposite sides of the same animal, since individual variations in susceptibility between different rabbits were often considerable. The preparation to be tested was infiltrated into the skin by a series of intracutaneous injections into a freshly shaven area about 3 to 4 cm. in diameter until 2 cc. had been injected. The animals were tested by intracutaneous inoculation, into the prepared areas, of 0.1 cc. of a salt solution dilution of the total surface growth of a standard sized blood agar slant, the dosage varying from 1/100 to 1/1000 of a slant. Doses of 1/500 to 1/250 of a slant gave the sharpest results. All inoculations made were with a single strain of *Staphylococcus aureus* isolated from a case of osteomyelitis, since this strain was the most virulent one for rabbits of those at our disposal. The majority of the filtrates were made from this same strain, but two others were also tried without variation in results. The test was usually made within 18 to 48 hours after the infiltration of the material.

1. Filtrate in One Area, Control Area Untreated.

TABLE I.

Rabbit No.	Date of infiltration.	Date of test.	Dose (fraction of one blood agar slant).	Results.	
				Protected area.	Control area.
1	Dec. 11	Dec. 12	1/500	±	++++
2	" 15	" 16	1/250	+++	++++
3	" 17	" 19	1/1000	+	++
4	Jan. 13	Jan. 15	1/1000	±	+++
5	" 14	" 15	1/1000	±	++

In this and the following tables ± indicates a lesion less than 0.5 cm. in diameter, without necrosis; +, a lesion from 0.5 to 1 cm. in diameter, without necrosis; ++, a lesion from 1 to 2 cm., with necrosis and definite purulent exudate; +++, a lesion from 2 to 3 cm. with necrosis and definite purulent exudate; ++++, lesions over 3 cm. in diameter, with marked necrosis and purulent exudate.

The difference between the infiltrated and uninfiltrated areas was always definite, although the actual size of the area varied considerably with the dosage and still more with the individual differences between rabbits.

2. Test of the Broth Alone.

Rabbit A was infiltrated in two areas, one with extract and one with hormone broth. 24 hours later these two areas and an untreated one were all inoculated with 1/500 of a slant.

Normal area..... +++
Extract broth area..... +
Hormone " " ..... ±

Both types of broth gave definite protection, the hormone broth more marked.

3. Filtrate in One Area, Extract Broth in Second. Control Untreated Area.

TABLE II.

Rabbit No.	Date of infiltration.	Date of test.	Dose.	Lesions.		
				Filtrate area.	Extract broth area.	Control area.
6	Jan. 16	Jan. 17	1/1000	+	+	Not done.
7	" 16	" 17	1/1000	+	+	" "
8	Feb. 3	Feb. 5	1/500	±	±	+++
9	" 3	" 5	1/250	+	+	+++
10	" 4	" 6	1/250	+	+	+++
11	" 4	" 6	1/250	+	+	+++

Both filtrate and extract broth produced a definite degree of immunity of the locality infiltrated, but in none of the animals could any significant difference in effectiveness between uninoculated broth and filtrate be noted. The lesions in the infiltrated areas were always small, indurated, and never showed more than a single drop of pus in the center at any stage. They generally healed without breaking down and discharging. In contrast, the normal areas developed large, purulent areas, with necrosis and sloughing at the center, and were surrounded by a wide border of erythema.

To see if variation in the infecting dose could bring out significant variation in effectiveness between broth and filtrate in a single animal, the following experiment was done. Three areas on each side of the animal were infiltrated intracutaneously, those on the left with extract broth, those on the right with filtrate.

TABLE III.

Rabbit No.	Dose.	Broth areas.	Filtrate areas.	Control area.
12	1/100	+++	+++	
	1/250	+	±	
	1/500	±	±	++

No significant differences between broth and filtrate appeared.

In further attempts to bring out possible differences between bacterial filtrates and broth, the time intervals were varied widely.

TABLE IV.

Rabbit No.	Interval since infiltration.	Dose.	Filtrate areas.	Broth areas.	Control areas.
	hrs.				
13	4½	1/500	±	±	+
14	8	1/500	±	±	++
15	12	1/250	±	±	+++
16	24	1/500	±	±	++
	48	1/500	±	+	
	72	1/500	+	+	
	days				
17	8	1/400	±	+	+++
	15	1/400	++	+	

At none of the intervals tested from 4½ hours up to 14 days could significant variation between broth and filtrate areas be shown.

4. Attempts to Immunize by the Transcutaneous Method with Moist Dressings Applied to the Freshly Shaven Skin.

Large areas were always shaved containing at least 100 to 200 sq. cm. A thick dressing of absorbent cotton and gauze, thoroughly saturated in the filtrate or broth, was firmly bound over the area and left in place for 24 hours. Separate rabbits were used for the broth and the filtrate dressings, and each rabbit was then tested in the treated area and in a freshly shaven one.

TABLE V.

Rabbit No.	Dressing.	Dose.	Treated area.	Control area.
18	Broth.	1/250	++	++
19	Filtrate.	1/250	++	++
20	Broth.	1/250	+++	+++
21	Filtrate.	1/250	+++	+++

The lesions in the treated areas, although not differing materially in size from the control lesions, were slightly more discrete and showed less surrounding border of erythema. No difference whatever between broth and filtrate areas was observed.

5. Reinoculation Experiments.

TABLE VI.

Rabbit No.	Length of time since first abscess was produced.	Dose.	Old areas.	Control areas.
	days			
22	24	1/250	±	+++
7	25	1/1000	±	+++
23	33	1/500	±	++
	28	1/250	±	++++
1	33	1/500	±	+++
	56	1/250	++	+++

Several areas where abscesses had completely healed, except for a small white scar, were reinoculated close to, but not into, the visible scar tissue, with the same strain of *Staphylococcus aureus*.

If inoculation is done very close (0.5 to 1.0 cm.) to the scar of a healed staphylococcus abscess, a definite local immunity is evident for at least 4 to 5 weeks. In one area tested after 8 weeks this had disappeared. If the inoculation is as much as 1.5 to 2 cm. from the visible scar an abscess of the ordinary size is produced.

No evidence of a general cutaneous immunity appeared, even in animals like No. 1, which received a total of eight reinoculations. To test this further, Rabbit 7 was reinoculated eleven times, the last nine injections being in groups of three at weekly intervals. He still developed an abscess of the usual size (+ + +) with a dose of 1/500 of a slant 1 week after the last three injections.

6. Inoculation of Healed Areas with Streptococci.

Healed sites of staphylococcus infections were tested with a strain of streptococci freshly isolated from a case of human erysipelas. It was not very virulent for rabbits but produced sharply defined areas of erythema and induration without necrosis or pus formation. A second strain isolated from a case of human septicemia proved more virulent and was used in the last three tests.

As in the reinoculation experiments with staphylococci, it was necessary to keep very close to the scar of the former abscess to demonstrate any immunity.

TABLE VII.

Rabbit No.	Length of time since first abscess was produced.	Dose.	Old areas.	Control areas.
	days			
4	35	1/10	±	++
6	32	1/20	±	±
13	25	1/10 (fresh strain).	±	++
19	25	1/10	±	+++
8	20	1/10	±	+++
9	20	1/10	±	++

DISCUSSION.

In the production of bacterial filtrates by Besredka's methods no evidence of specific growth inhibition was observed in any preparation, even in those reinoculated and refiltered as many as four times. Where any growth inhibition at all occurred, it was entirely non-specific, and a simple correction of the hydrogen ion concentration was always sufficient to promote luxurious regrowth of the specific strain. Besredka's published data are so incomplete that it is impossible to judge whether his growth-inhibitory factor was a wholly non-specific one, such as hydrogen ion concentration, or exhaustion of some simple nutritive constituent, or whether he may possibly have been dealing with a bacteriophage phenomenon.

That such filtrates do produce a strictly local immunity of the skin when injected intracutaneously our results confirm, but we could never by any variation of age of filtrate, inoculating dose, or of interval between skin infiltration and test inoculation show any greater protection from the filtrate than from the broth with which it was made. In fact, in several instances the original or uninoculated sterile broth seemed slightly more effective. The method of poulticing the skin with moist dressings proved wholly ineffective. That such treatment can produce a general immunity of the entire animal, as Besredka claims, seems most improbable, especially since multiple and repeated cutaneous infection with virulent organisms failed to give any evidence of a general immunity. Rivers' results, as well as our own, show that the local immunity from either broth or bacterial

filtrate injection of the skin is entirely non-specific in character. We have no direct evidence of its character, but are inclined to agree with him that it is most probably due to a rapid mobilization of phagocytes. This would also probably explain the strictly local and non-specific immunity persisting for several weeks in the site of the former infectious lesions of the skin.

SUMMARY.

1. Bacterial filtrates were prepared by the methods described by Besredka, but these never developed any specific growth-inhibiting factors.

2. Such filtrates produced a strictly local immunity of the areas of the skin infiltrated but such protection was never greater than that produced with the broth from which they were prepared. This immunity was manifest within 8 hours and persisted at least 15 days.

3. Transcutaneous treatment by moist dressings soaked in either filtrate or broth were entirely ineffective.

4. Sites of healed cutaneous lesions show a strictly local and non-specific immunity for a period of 5 to 6 weeks.

5. Repeated intracutaneous infection with virulent staphylococci failed to produce a general cutaneous immunity.

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1. Besredka, A., *Compt. rend. Soc. biol.*, 1923, lxxxviii, 1273; lxxxix, 7. Besredka, A., and Urbain, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 506. Besredka, A., *Ann. Inst. Pasteur*, 1924, xxxviii, 565.
2. Rivalier, E., *Compt. rend. Soc. biol.*, 1923, lxxxix, 711.
3. Rivers, T. M., *J. Exp. Med.*, 1925, xli, 179. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1925, xli, 185.

BIOLOGY OF BACTERIUM LEPISEPTICUM.

II. The Structure of Some Iron Compounds Which Influence the Growth of Certain Bacteria of the Hemophilic, Anaerobic, and Hemorrhagic Septicemia Groups.

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In a previous report, one of us has shown that certain virulent cultures of *Bacterium lepisepticum* need, for optimum growth and maintenance, either a plain nutrient medium at a reduced oxygen tension or, under aerobic conditions, this same medium plus rabbit blood.[1] The blood was shown to function in high dilutions to the limit of the benzidine reaction, and also after autoclaving for 30 minutes at 115 pounds pressure. For these reasons it was concluded that the active substance contained in blood could not be a food, nor of vitamine nature. But its behavior did suggest a function similar to that of the X factor, the presence of which Avery and his associates[2] regard as one of the essentials for optimum growth of hemophils, anaerobes, and pneumococci at atmospheric pressure.

Avery has described the X factor as follows: "The so called X factor, which is associated with the pigment fraction of blood, gives the peroxidase reaction, and is not destroyed by moist heat at 120°C. . . . . Moreover, the X substance is active in such minute amounts as to suggest that it functions as a biocatalyst."[3] It would seem then that the principle needed for good growth of virulent strains of *Bacterium lepisepticum* resembles the X factor.

The X factor has been found in plant tissue[2] and in a solution of ferrous sulfate and gum arabic.[4] A list of substances which function as X has been assembled

[1] Webster, L. T., *J. Exp. Med.*, 1925, xli, 571.

[2] Avery, O. T., *et al.*, *J. Exp. Med.*, 1921–24, xxxiv, xxxviii–xl.

[3] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 296.

[4] Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 59.

from the literature by Novy.[5] The chemical constitution of most of them is, however, so little understood that it seemed advisable to test the action of simple compounds in the hope that additional facts relating to the structure and behavior of the X mechanism might be recognized.

Technique.

A number of substances with some of the properties of hemoglobin have been used; sodium pentacyano-aquo-ferroate and its derivatives, ferrous and ferric oxides, and vegetable charcoal.

The peroxidase reaction was carried out with either benzidine chloride dissolved in distilled water, or benzidine dissolved in glacial acetic acid, plus superoxol (hydrogen peroxide, Merck). The presence of ferrous iron was detected by the Prussian blue reaction, or by titration with $KMnO_4$. The amount of oxygen absorbed by a compound was determined by means of a Barcroft-Haldane apparatus as modified by Brodie.[6]

The biological test consisted in adding the given substance to tubes containing 5 cc. of plain meat extract broth, pH 7.4, and inoculating each with *Bacterium lepisepticum*, Type D, in numbers varying exponentially from 10^9 to 10^0. If growth occurred in tubes seeded with less than 100,000 bacteria, and if, after 3 or more weeks incubation at 37°C., no Type G variants appeared in any culture, the test was considered positive and the compound was considered to function as the X factor.[1]

Sodium Pentacyano-Aquo-Ferroate.

Baudisch's studies of the catalytic properties of iron[7] led us to test the activity of sodium pentacyano-aquo-ferroate, an inorganic compound of known formula discovered by Hofmann.[8] This substance, like hemoglobin, contains a ferrous radical, gives a strong peroxidase reaction, and absorbs oxygen at 37.5°C., at an initial rate of 2.38 cc. per gm. per hour.[9] It is derived from the inactive sodium ferrocyanide by the replacement of one NaCN group by H_2O. This H_2O group,

[5] Novy, F. G., Jr., *J. Infect. Dis.*, 1925, xxxvi, 343.

[6] Brodie, T. G., *J. Physiol.*, 1909–10, xxxix, 391.

[7] Baudisch, O., and Welo, L. A., *J. Biol. Chem.*, 1924, lxi, 261.

[8] Hofmann, K. A., *Ann. Chem.*, 1900, i, 312.

[9] We are indebted to Dr. James A. Hawkins for measuring the oxygen absorption of these compounds. His readings for sodium pentacyano-aquo-ferroate were as follows:

Cell 14 = −2.720 cc. O_2 consumed per gm. per hour.
" 23 = −2.010 " O_2 " " " " "
" 13 = −2.41 " O_2 " " " " "

although forming an integral part of the molecule, is associated with an available bond which endows the central iron atom with activity, catalase, oxygenase, and peroxidase properties.

Experiment 1.—Nine tubes containing 5 cc. of plain extract broth, pH 7.4, plus 1 cc. of a 10 per cent sterile solution of the aquo salt, nine tubes of broth plus 1 cc. physiological salt solution, and nine tubes of broth plus 1 cc. of a 10 per cent solution of autoclaved, laked rabbit corpuscles were inoculated with a 17 hour broth culture of *Bacterium lepisepticum*, Type D, in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation the cultures were examined for growth and later for the appearance of Type G variants.

The results are shown in Table I. Growth occurred in all the aquo salt and blood tubes, including those which had received the smallest

TABLE I.

Effect of Sodium Pentacyano-Aquo-Ferroate on the Growth of Bacterium lepisepticum, Type D.

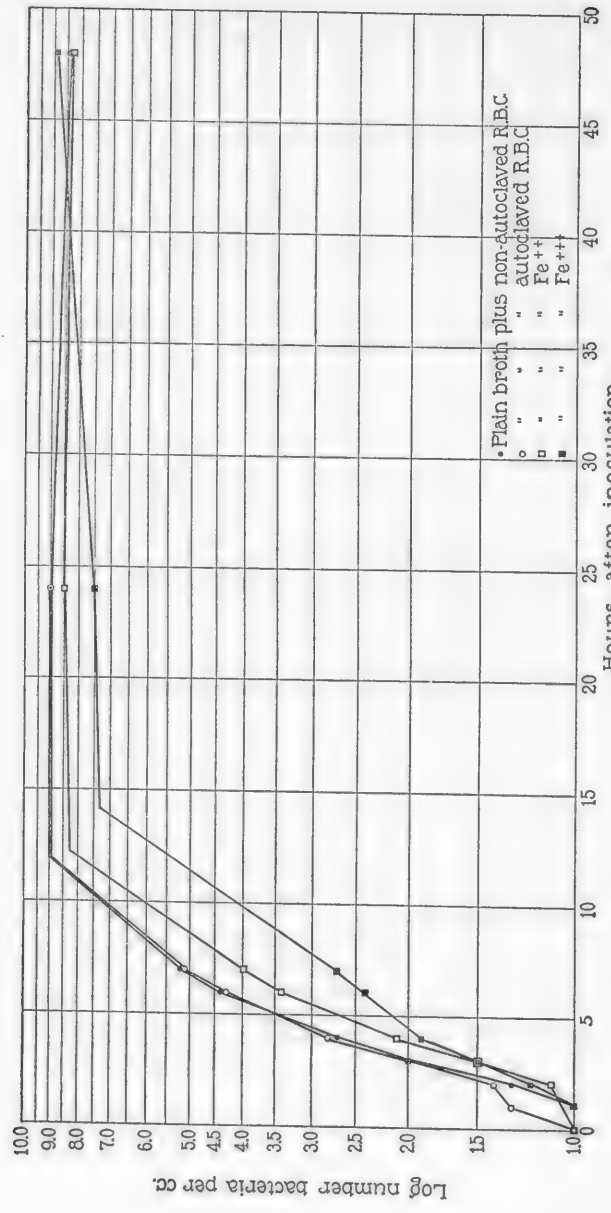
Medium.	Benzidine test.	Reducing power.	Oxygen-absorbing power.	No. of bacteria per tube.									Type G variation.
				10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}	
Plain broth.....	0			++	++	++	0	0	0	0	0	0	++
Plain broth + laked blood corpuscles....	++	++	++	++	++	++	++	++	++	++	++	++	±
Plain broth + aquo salt.....	++	++	++	++	++	++	++	++	++	++	++	++	±

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

number of organisms, but only in such of the plain broth tubes as had been seeded with 100,000 or more. Later, Type G variants appeared in these cultures, but not in the blood or aquo salt tubes.

Further experiments demonstrated that this aquo salt was active as the X factor in dilutions of 1/100,000 gm. per cc. This dilution was also the limit of the benzidine reaction. The ferric salt was found to be active as well, but the presence of NO_2 and NH_3 groups in place of H_2O decreased its sensitivity.

Experiments with strains of *Bacillus avisepticus* yielded similar results.



TEXT-FIG. 1. Growth of *Bacterium lepisepticum*, Type D, in plain broth plus aquo salt.

In the work which followed a determination of the rate of bacterial growth was made by the serial counting method.[1]

Experiment 2.—To one Erlenmeyer flask containing 150 cc. plain broth was added 18 cc. hemolyzed, washed rabbit blood corpuscles plus 12 cc. 0.85 per cent NaCl; to another, 20 cc. autoclaved red cells plus 10 cc. saline; to a third, 30 cc. of an aqueous 10 per cent solution of the aquo ferrous salt, and to a fourth, 30 cc. of the aquo ferric salt. Samples from the first three flasks gave strong benzidine reactions; in Flask 4 the test was weaker.

Each flask was now inoculated with 1 cc. of a 1:1,000,000 dilution of a 17 hour plain broth culture of *Bacterium lepisepticum*, Type D, and incubated at 37°C. Counts were made at half hour intervals for the first 6 hours; then at 24 and 48 hours. The numbers of bacteria per cc. in each flask at various time intervals are plotted in Text-fig. 1.

Following an inoculation of less than 10 bacteria per cc., the count in each flask increased immediately and logarithmically to reach a maximum of about 1 billion per cc. within 24 hours. The growth curves of the bacteria in the flasks containing broth and blood, and broth and aquo ferro salt paralleled one another closely; that from the ferric salt medium differed in that the rise was somewhat less abrupt. Evidently the aquo salt, by supplying the X factor, furnishes optimum conditions for the aerobic growth of *Bacterium lepisepticum.*

Experiment 3.—This experiment was planned to test the activity of the aquo ferrous salt as the X factor in the growth of organisms of the so called hemophilic group.

Three cultures were obtained from Dr. T. M. Rivers. The first, *B. influenzæ* No. 6,[10] required for growth the presence of the X and V factors; the second, "J. R. L.,"[11] needed the V factor alone; and the third, "Dog,"[12] the X factor only. These strains were taken from blood agar slants and inoculated into 10 per cent rabbit blood broth tubes. After 24 hours incubation, 0.1 cc. from each tube was transferred to a tube containing 4 cc. of plain broth, pH 7.4, plus 1 cc. of a 10 per cent solution of the aquo salt. The media gave a strong benzidine test prior to inoculation. These first subcultures were then incubated overnight and on the following day 0.1 cc. was plated on chocolate agar to determine the presence of live organisms, and one loopful was transferred to the second series of aquo salt broth tubes.

This procedure was continued for six subcultures, each after an incubation period of 24 to 48 hours. Then 0.1 cc. of each culture was transferred to tubes of

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[10] Rivers, T. M., and Poole, A. K., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 202.

[11] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 429.

[12] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 149.

TABLE II.

Growth of Influenza Bacilli in Media Containing Aquo Salt.

Culture.	Subculture. No.	Aquo salt.	Control plates.	Aquo salt + yeast.	Subculture No.	Control plates.	Yeast.	Subculture No.	Control plates.	Blood.	Control plates.	Plain broth.
Infl.										++	++	0
J. R. L.										++	++	0
Dog.										++	++	0
Infl.	1	±	+									
J. R. L.	1	±	+									
Dog.	1	++	++									
Infl.	2	±	+									
J. R. L.	2	±	+									
Dog.	2	++	++									
Infl.	3	±	+									
J. R. L.	3	±	+									
Dog.	3	++	++									
Infl.	4	±	+									
J. R. L.	4	±	+									
Dog.	4	++	++									
Infl.	5	±	+									
J. R. L.	5	±	+									
Dog.	5	++	++									
Infl.	6	±	+									
J. R. L.	6	±	+									
Dog.	6	++	++									
Infl.	7	±	+	++	1	++	++	1	++	++	++	0
J. R. L.	7	±	+	++	1	++	++	1	++	++	++	0
Dog.	7	++	++	++	1	++	++	1	++	++	++	0
Infl.	8	±	+	++	2	++	0	2	0	++	++	0
J. R. L.	8	++	++	++	2	++	++	2	++	++	++	0
Dog.	8	++	++	++	2	++	±	2	+	++	++	0
Infl.	9	±	+	++	3	++	0	3	0	++	++	0
J. R. L.	9	±	+	++	3	++	++	3	++	++	++	0
Dog.	9	++	++	++	3	++	0	3	0	++	++	0

±, survival, very slight growth; +, moderate growth; ++, good growth.

aquo salt broth, aquo salt plus 1.0 per cent yeast extract broth, and 1.0 per cent yeast extract broth. Blood and plain broth tubes served as controls. Plates were smeared the next day and subcultures were made from each tube to a second series of similar media. Three consecutive transfers were carried out in this manner.

Table II shows the results. The Dog culture grew well in aquo salt broth, but *Bacillus influenzæ* and J.R.L., although surviving for nine subcultures, grew very poorly. All three cultures grew profusely in broth containing aquo salt plus yeast extract, and only J.R.L. grew in the media containing yeast extract alone. From these experiments it was concluded that the aquo salt functions as the X factor in the growth of *Bacillus influenzæ* and other hemophils as well as in the case of bacteria of the hemorrhagic septicemia group.

Strains of *Bacillus welchii* and *Bacillus sporogenes* which failed to grow in tubes of aerobic broth sealed with vaseline, but which did multiply luxuriantly when the oxygen tension in the broth was reduced mechanically by boiling and sealing, showed similar abundant growth when aquo salt was added to aerobic broth sealed with vaseline.

Further experiments were made with oxides of iron which were being studied by Welo and Baudisch.[13] Fe_3O_4, natural magnetite, was first tried and, although quite insoluble, was found to give a positive benzidine reaction and to function as the X principle for the growth of *Bacterium lepisepticum.*

Iron Oxides.

Fe_3O_4, Lefort's oxide, was then made by precipitating one part $FeSO_4$ and one part $Fe_2(SO_4)_3$ with NaOH. This substance possesses a definite cubic crystal structure, a magnetic susceptibility of 0.152, absorbs oxygen at an initial rate of 0.0043 cc. per gm. per hour,[14]

[13] Welo, L. A., and Baudisch, O., *J. Biol. Chem.*, 1925, lxv, 215.

[14] Dr. Hawkins' readings for the oxides were the following.

Fe_3O_4 Cell 14 = 0.0045 cc. O_2 consumed per gm. per hour.
" 23 = 0.0043 " O_2 " " " " "
" 13 = 0.0044 " O_2 " " " " "

Fe_2O_3 active Cell 14 = 0.0072 cc. O_2 consumed per gm. per hour.
" 23 = 0.0092 " O_2 " " " " "
" 13 = 0.0076 " O_2 " " " " "

Fe_2O_3 inactive Cell 14 = 0
" 23 = 0
" 13 = 0

and gives a positive benzidine reaction in broth. Oxidation at 330° removed all trace of ferrous iron, but did not affect the crystal structure. The magnetic susceptibility and initial rate of oxygen absorp-

TABLE III.

Effect of Ferric Oxides on the Growth of Bacterium lepisepticum, Type D.

Medium.	Benzidine.	Ferrous iron.	Initial rate of oxygen absorption.	No. of bacteria per tube.									Type G variants.
				10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}	
			cc. per gm. per hr.										
Plain broth....	0	0	0	++	++	++	++*	0	0	0	0	0	++
Plain broth + blood.....	++	++	–	++	++	++	++	++	++	++	++	++	±
Plain broth + Fe_3O_4.....	++	++	0.0043	++	++	++	++	++	++	++	++*	0	±
Plain broth+ active Fe_2O_3.	++	0	0.008	++	++	++	++	++	++	++	+*	0	±
Plain broth+ inactive Fe_2O_3.....	0	0	0	++	++	++	++*	0	0	0	0	0	++

–, no determination.

* Growth after 48 hours incubation.

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

tion increased slightly.[14] The benzidine test continued positive. Further heating to 550° changed the crystal structure unit to a rhombohedral form, destroyed its magnetic and oxygen-absorbing properties,[14] and its power to oxidize benzidine. Catalase activity was present before and after heating.

The biological test was carried out on these three oxides as follows:

Experiment 4.—About 0.5 gm. of each of these substances, Lefort's oxide (Fe_3O_4), active Fe_2O_3, and inactive Fe_2O_3, was added to each of nine tubes containing 5 cc. of plain broth, pH 7.4. These three series together with a control set of plain broth and blood broth tubes were inoculated with a 17 hour plain broth culture of *Bacterium lepisepticum* in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation they were examined for growth. Later the variant G forms were sought for by plating methods.

Table III shows the results of this experiment. Growth occurred in every tube to which blood, Fe_3O_4, or active Fe_2O_3 had been added, and no G variants appeared for 2 weeks. In the plain broth and inactive Fe_2O_3 series bacterial multiplication occurred only in those tubes which had received an inoculation of 100,000 organisms or more. G forms were abundant in these after 72 hours. Further quantitative tests showed that at least 0.1 gm. of the active oxides per cc. of broth was necessary for a positive reaction.

Bacillus avisepticus behaved in a similar manner. The hemophilic and anaerobic strains used in the previous experiments grew well in the presence of the active Fe_2O_3, while no growth occurred when the inactive oxide was used.

DISCUSSION.

Two types of inorganic iron compounds of known structure have been found to show the so called X type of biocatalytic activity, (1) pentacyano iron salts and (2) certain iron oxides. In each instance definite physical and chemical properties may be related to the growth-promoting power. Sodium pentacyano-aquo-ferroate contains an unsaturated bond which is associated with oxygen-absorbing and peroxidase properties, as well as with growth-promoting activity. And Fe_3O_4, although quite insoluble, has similar characteristics. When this latter substance is heated just enough to oxidize all ferrous iron, a form of Fe_2O_3 is obtained which retains the same crystal structure, oxygen-absorbing, peroxidase, and growth-activating properties. Further heating of this Fe_2O_3 alters its crystal structure and destroys its oxygenase, peroxidase, and biocatalytic action.

The contrast in growth-promoting power of these two similar iron oxides indicates anew the close association of this property with that

of oxygen absorption and of benzidine oxidation. Avery regards the iron-containing X substances as peroxidases which destroy metabolic, toxic, organic peroxides, and which, in some more subtle way, activate cell respiration. Novy, on the other hand, considers that the mechanism of all chemical, animal, and vegetable X substances is to reduce the oxygen tension of the medium to a level suitable for growth.[5]

The data so far accumulated indicate that, though many X substances have characteristics in common with respiratory ferment, the only property common to all is that of being able to absorb oxygen.

The effect of these biocatalysts on the appearance of bacterial variants should be noted. Their presence in the media insures optimum growth and inhibits variation, while without them growth is retarded and variants are abundant after 48 hours. Apparently the occurrence of this type of bacterial variation is intimately associated with the presence of excess oxygen in the medium.

CONCLUSIONS.

Inorganic iron substances of known chemical structure have been tested for their ability to function as the X factor in the growth of bacteria of the hemophilic, anaerobic, and hemorrhagic septicemia groups.

Each iron compound possessing this biocatalytic activity showed oxygen-absorbing and peroxidase properties.

The interrelation of these mechanisms is discussed.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

III. Some of the Factors Determining the Number and Size of Plaques of Bacterial Lysis on Agar.

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Plate 20.

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The quantitative evaluation of the activity of the so called bacteriophage is usually accomplished by one of two methods.

The earlier procedure and its modifications[1,2] make use of the appearance of clear spots—"*taches vierges*" or "plaques"—in the midst of an even growth when bacteria are seeded on agar in the presence of bacteriophage-containing solutions. It is assumed that each clear spot represents a colony of "bacteriophage," and the count of these colonies is carried out in a manner analogous to that used in counting colonies of bacteria.

The other method[3,4] consists in preliminary serial dilution of the bacteriophage-containing fluid in sterile broth, with subsequent inoculation of these serial dilutions with a standard suspension of susceptible bacteria. In this case it is assumed that the highest dilution in which the lysis of bacteria occurs after a proper incubation must have received at least one unit of lytic principle and thus the total number of active units present in a given volume of the original fluid may be computed.

If in the case of "bacteriophage" one is dealing with a suspension of discrete living elements, as assumed by d'Hérelle, the count of these particles by both methods should be consistent, as it is when used in enumerating bacteria, and therefore the methods could be used inter-

[1] d'Hérelle, F., Le bactériophage, son rôle dans l'immunité, Monographies de l'Institut Pasteur, Paris, 1921, 17.

[2] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 12.

[3] Appelmans, R., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1098.

[4] Werthemann, A., *Arch. Hyg.*, 1922, xci, 255.

changeably. However, the observations of several investigators,[5-8] as well as our own experience,[9] have shown that the lytic titers of bacteriophage-containing fluids are considerably higher when determined by the broth dilution method than on counting the plaques on agar. Before going into the reason for this discrepancy, we have felt that it is necessary to establish first whether the lytic principle is really particulate, as assumed by d'Hérelle, or whether, on the contrary, it is present in a state of true solution.

Particulate Distribution of the Lytic Principle.

When a broth culture of young, susceptible bacteria is allowed to grow in the presence of suitable amounts of the active lytic principle, the concentration of the latter can be shown to increase rapidly during the period of logarithmic growth of bacteria. After 18 to 24 hours of incubation the concentration of the lytic principle usually reaches its highest level, after which it remains unchanged, irrespective of further changes in the bacterial count. If at the time bacteria are removed by filtration, the filtrate is distributed in a series of broth tubes in gradually diminishing amounts, from 0.1 cc. down, and if each of the tubes of the series is seeded with 0.1 cc. of a suspension of young culture (18 hours) containing about 1,000,000,000 of susceptible bacteria per cc., one finds that after a suitable period of time (18 to 24 hours) lysis of bacteria has taken place in all tubes receiving more than 10^{-10} cc. of the filtrate. All the tubes receiving less than 10^{-10} cc., with but few exceptions, show no lysis. Moreover, if the tubes are incubated further for 18 to 24 hours, and if at this time their contents are heated, or filtered, to kill, or remove, the secondary growth of resistant bacteria, and titrated for lytic activity by the serial dilution method,[3,4] one finds that the concentration of the lytic principle is at the same maximum in all the tubes in which lysis was observed

[5] Doerr, R., *Schweiz. med. Woch.*, 1923, iv, 1009.

[6] Doerr, R., and Rose, G., *Schweiz. med. Woch.*, 1924, v, 10.

[7] Reichert, F., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 235.

[8] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 228.

[9] Bronfenbrenner, J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 315.

earlier, irrespective of the amount of lytic filtrate they received originally. On the other hand, all the tubes receiving less than 10^{-10} cc. of the filtrate show no lytic activity.

The results of the experiment illustrated in Protocol 1 suggest that the lytic agent is present in the filtrate not in the form of true solution, but in a particulate state. Thus, the fact that 10^{-10} cc. of the filtrate produces lysis, whereas 10^{-11} cc. produces no lysis is explained as showing that 10^{-10} cc. contains at least one and less than ten indivisible active particles of bacteriophage. Since the distribution of such discrete particles in very high dilutions may be such that not every cc. of dilution will contain a particle, it is scarcely surprising that in the next dilution (1×10^{-11} cc.) we find two tubes only (Nos. 2 and 8) showing lytic activity.

If, then, it is true that 1×10^{-10} cc. contains one indivisible particle of the active principle, it should follow that one-half or one-third of this amount will contain less than one, and hence show no lytic action. If, however, the lytic agent is present in the form of a true solution, combination of three portions of $1/3 \times 10^{-10}$ cc. should result in the same activity as is produced by one whole portion of 1×10^{-10} cc. Or, the lytic agent may be present in a measurable amount in even 1×10^{-11} cc., which concentration, however, would appear too low to induce a change in bacteria resulting in their lysis. In this event a combination of 10 portions of 1×10^{-11} cc. should be active. Should this prove to be the case, the true solubility, as opposed to particulate distribution of bacteriophage, would be established. The following experiment is one of a series of similar ones carried out to obtain information on this point.

Active filtrate Laudman Shiga was diluted with sterile broth so that each cc. of dilution contained 1×10^{-9} cc. of the original filtrate. 2 cc. of this dilution was added to 198 cc. of sterile broth, mixed thoroughly, and distributed in 10 cc. amounts in each of twenty sterile tubes. Thus each of the tubes of the series received 1×10^{-10} cc. of the original filtrate (Protocol 2, *A*). In a similar manner another series of twenty tubes was prepared to contain in each 1×10^{-11} cc. of the original filtrate (Protocol 2, *B*). Each of the tubes was then seeded with 0.1 cc. of a suspension of *Bacillus dysenteriæ* Shiga containing 10^9 susceptible bacteria per cc. and incubated at 37°C. The appearance of lysis was recorded at the end of 18 hours, as well as at the end of 40 hours of incubation. At this time all the tubes were heated to destroy the bacteria, and a drop of the contents was deposited on a

*Protocol 1.**

Increase of Lytic Principle to a Maximum.

Amount of active filtrate in 0.1 cc. volume, *cc.*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	0
Broth, *cc.*	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.9
Bacterial suspension, *cc.*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubated at 37° C. for 18 to 24 hrs.													
Results	+	+	+	+	+	+	±	±	±	±	−	−	−
Incubated again for 18 hrs. at 37°C., heated for 30 min. at 56°C. (or filtered), and titrated.													
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Results of titration. 1×10^{-1} cc.	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-2} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-5} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-7} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-9} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-10} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-11} "	−	+	−	−	−	−	−	+	−	−	−	−	−
1×10^{-12} "	−	−	−	−	−	−	−	−	−	−	−	−	−

* In this as well as in following protocols the sign + signifies clearing of culture (or lysis of bacteria); the sign ± signifies a partial clearing; and the sign − signifies absence of lysis.

surface of agar, previously seeded with susceptible bacteria (agar transfer). This procedure served as a final check on the lytic activity of the contents of each tube.

Protocol 2.

Particulate Distribution of the Lytic Agent.

Series A.

2×10^{-9} cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-10} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No.....	1	2	3	4	5	6	7	8	9	10
Lysis.....	±++	±++	±++	±++	−−−	±++	±++	±++	−−−	±++
Tube No.....	11	12	13	14	15	16	17	18	19	20
Lysis.....	−−−	±++	±++	±++	±++	±++	±++	±++	−−−	±++

Contents of Tubes 5, 9, 11, and 19 were now combined, filtered, and the filtrate was seeded with susceptible bacteria. No lysis was observed.

Series B.

2×10^{-10}cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-11} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No.....	1	2	3	4	5	6	7	8	9	10
Lysis.....	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	±++	−−−
Tube No.....	11	12	13	14	15	16	17	18	19	20
Lysis.....	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−

Contents of Tubes 1 to 8 and 10 to 20 were all combined, filtered, and the filtrate was seeded with susceptible bacteria, but no lysis could be observed.

As will be observed from the results presented in Protocol 2, a great majority of the tubes of Series A, each containing 10^{-10} cc. of the original filtrate, showed lysis, as did also a few of the tubes in Series B.

Protocol 3.

Effect of the Concentration of Agar on Lytic Activity.

5 per cent beef infusion agar, *cc.*	9	9	9	5	5	5									
2 per cent beef infusion agar, *cc.*							5	5	5	2.5	2.5	2.5	1.5	1.5	1.5
Beef infusion broth, *cc.*	0.0	0.0	0.0	4	4	4	4	4	4	6.5	6.5	6.5	7.5	7.5	7.5
B. dysenteriæ Shiga suspension, *cc.*	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
0.1 cc. Duggan Shiga bacteriophage diluted in H_2O	$1:10^4$	$1:10^6$	$1:10^8$	$1:10^4$	$1:10^6$	$1:10^8$	$1:10^7$	$1:10^8$	$1:10^9$	$1:10^7$	$1:10^8$	$1:10^9$	$1:10^7$	$1:10^8$	$1:10^9$
Resulting agar concentration, *per cent*	4.5	4.5	4.5	2.5	2.5	2.5	1.0	1.0	1.0	0.5	0.5	0.5	0.3	0.3	0.3
Incubated at 37°C. overnight.															
Plaque count	0	0	0	15	0	0	8	1	0	39	5	0	0	75	7	0
Maximum diameter of plaques, *mm.*	—			0.25	—	—	1	—	—	2.5	—	—	4	—	—	
No. of plaques per cc. (titer).				($\pm 15 \times 10^5$) 0.00015 x 10^{10}			± 0.1 x 10^{-10}			± 0.5 x 10^{10}			± 0.7 x 10^{10}			
No. of units per cc. as determined by broth titration																1×10^{10}

The contents of the tubes showing lysis were discarded and those showing absence of lytic activity were now combined, filtered, seeded with susceptible bacteria, and incubated at 37°C.

Since combination of the contents of several tubes containing insufficient amount of lytic principle in each does not result in bringing about lysis, it appears that the lytic principle is distributed through the medium in the state of indivisible units.

Effect of Concentration of Agar on Lytic Titer.

The results of the preceding experiment indicate that the lytic agent is distributed in the medium as if particulate. If these particles are living and multiplying individuals subject to numerical evaluation, as suggested by the early work, their count by dilution in broth should correspond with that obtained on agar. However, as stated above, this is not the case.

While investigating to explain this discrepancy, it occurred to us that the ease with which lytic principle is known to be adsorbed on a variety of colloidal substances [10–12] may be responsible for the lowering of lytic activity when tested on agar. It seemed logical to suppose that by decreasing the agar content of the medium one might cause the plate counts to approach more nearly the values obtained by parallel broth dilution titrations. Accordingly, experiments were performed in which anti-Shiga as well as anti-*coli* lytic filtrates were plated out on agar, varying in concentration from about 4.5 per cent to 0.25 per cent. In parallel titrations the lytic activity of these filtrates was also determined by the broth dilution method. Protocol 3 outlines one of many of these experiments.

The lytic filtrate Duggan Shiga used in this experiment when tested in broth caused lysis in the amount of 1×10^{-10} cc. Thus, as seen from Protocol 3, when the concentration of agar is reduced below 0.5 per cent, the count of plaques per cc. obtained thereon is 7×10^9 or practically that obtained by the broth dilution method (1×10^{10}). On the other hand, when the concentration of agar is raised to 1 per

[10] Doerr, R., *Klin. Woch.*, 1922, i, 1489.

[11] Nakamura, O., *Arch. Hyg.*, 1923–24, xcii, 61.

[12] Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.

cent, the readings are only 10 per cent of those obtained by broth titration, and if the concentration of agar is further increased, the titer becomes progressively lower.

Effect of Agar Concentration on the Plaque Size.

In addition to the changes in the number of plaques observed as the concentration of agar was varied, we found that in general the average size of plaques tended to increase as the concentration of agar

Protocol 4.

Effect of the Concentration of Agar on the Activity of Purified Lytic Principle.

Agar concentration, *per cent*.....	4.5	2.5	1	0.5
Amount of agar, *cc*.....	9	9	9	9
18 hr. culture of *B. dysenteriæ* Shiga, *cc*.....	0.9	0.9	0.9	0.9
0.1 cc. of Duggan Shiga filtrate diluted.....	$1:10^1$	$1:10^3$	$1:10^5$	$1:10^{10}$
Plaque count.....	0.0	130	180	281
No. of plaques per cc. (titer)	0.0	(130×10^4) 0.00013×10^{10}	(180×10^6) 0.018×10^{10}	(281×10^7) 0.28×10^{10}
Maximum diameter of plaques, *mm*.....	—	0.25	1	2.5

Plates from which the above figures were taken are shown in the photograph in slightly less than actual size (Figs. 1 to 4).

was reduced (Protocol 3). This phenomenon seemed to us of great interest since it suggests that the size of the plaques may be not only a function of a given lytic agent, as is usually believed, but it may also depend on the concentration of agar. Since the lytic agents used by us in these experiments were recently isolated by us from fecal material, they may conceivably have consisted of mixtures of different lytic agents which could account for the lack of constancy in the size of plaques.[13–18] We therefore purified these filtrates by repeated

[13] Gratia, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 821, 824.

[14] Bail, O., and Watanabe, T., *Wien. klin. Woch.*, 1922, xxxv, 169.

[15] Bruynoghe, R., and Appelmans, R., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 96.

[16] Wolff, L. K., and Janzen, J. W., *Ann. Inst. Pasteur*, 1923, xxxvii, 1064.

[17] Wagemans, J., *Arch. internat. pharmacod. et thérap.*, 1923–24, xxviii, 159, 181.

[18] Matsumoto, T., *Wien. klin. Woch.*, 1923, xxxvi, 759.

passages on agar, each time from a single plaque; when the lytic agents thus obtained appeared to give plaques of reasonable uniformity, we replated them on agar of varying concentrations (Protocol 4).

Similar changes in the size and number of plaques under the influence of variations in concentration of agar were observed with purified lytic filtrates (Phage B. W. and Pet. 2 respectively) sent to us by Gratia.[13]

When Irish moss was substituted for agar, results similar to those shown above were obtained. As the concentration of jelly was reduced the size and the number of plaques were increased.

Protocol 5.

Effect of an Excess of Susceptible Bacteria on the Size of Plaques.

Molten 2 per cent agar, *cc.*	9	9	9	9	9	9
B. dysenteriæ Shiga 18 hr. culture, No. of bacteria in 1 cc.	10^{10}	10^{9}	5×10^{8}	1×10^{8}	1×10^{7}	1×10^{6}
0.1 cc. Laudman Shiga filtrate diluted	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}
Plates poured and incubated at 37°C. overnight.						
Maximum size of plaques, *mm.*	0.25	0.5	0.5	0.75	Unsatisfactory discontinuous growth.	

Effect of the Number of Susceptible Bacteria on the Number and Size of Plaques.

Since it is known that bacteriophage principle does not multiply except in the presence of bacteria, and, moreover, since it is generally assumed that the development of plaques is the result of lysis of bacteria surrounding or developing on the spot in close proximity with the particles of active agent, it is possible that the number and size of such plaques will also depend on the density of bacterial suspension subjected to lysis. This relation was studied by pouring a series of agar plates in which, all other factors being constant, the total number of susceptible bacteria was varied.

As can be seen from the results presented in Protocol 5, when the number of bacteria added was less than 100 million per cc., the growth was discontinuous and the plaques were not clearly demarcated. As

the number of bacteria increased, the size of the plaques diminished, the total count of plaques not being appreciably affected.

Effect of Relative Concentration of Young and Old Bacteria.

It is known that the lytic agent increases in titer only in the presence and at the expense of young susceptible bacteria; moreover, older bacteria are known to adsorb lytic agent without undergoing lysis, thus decreasing the concentration of free lytic agent in solution.[19–21]

Protocol 6.

Effect of Relative Number of Old and Young Susceptible Bacteria on Number and Size of Plaques.

Molten 1 per cent agar, *cc.*	9	9	9	9	9	9	9
18 hr. culture of *B. dysenteriæ* Shiga, *cc.*	1.0	0.75	0.5	0.25	0.0	1	0.0
12 day culture of *B. dysenteriæ* Shiga, *cc.*	0.0	0.25	0.5	0.75	1.0	0.0	1
0.1 cc. of Laudman Shiga filtrate diluted.	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0
Plates poured and incubated at 37°C. overnight.							
Maximum size of plaques, *mm.*	1	1	0.8	0.6	Discontinuous growth.	Even growth.	Discontinuous growth.
No. of plaques	240	105	82	20	Counting impossible.		

For these reasons it was thought that the relative concentration of young and old bacteria might also appreciably affect the appearance of plaques.

In order to investigate this question, emulsions of equal turbidity were prepared by suspending in physiological salt solution agar cultures of *Bacillus dysenteriæ* Shiga of 18 hours and of 12 days respectively. Varying amounts of each were added to the mixture of agar and lytic agents as indicated in Protocol 6.

[19] Adsorption experiment will appear in *The Journal of Experimental Medicine.*

[20] Jaumain, D., and Meuleman, M., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 362.

[21] Prausnitz, C., and Firle, E., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 148.

This experiment shows that both the number and the size of plaques diminish as the proportion of old bacteria is increased. When old bacteria only were used, the growth was discontinuous and the plaques could not be distinguished.

Effect of Presence of Resistant Bacteria on the Appearance of Plaques.

Since, in the preceding experiment, it was found that the presence of old bacteria affects the appearance of plaques, it was thought useful to inquire into the effect of young resistant bacteria. For this pur-

Protocol 7.

Effect of Relative Number of Susceptible and Resistant Bacteria on the Number and Character of Plaques.

Molten agar 1 per cent, *cc.*	9	9	9	9	9	9	9
B. dysenteriæ Shiga susceptible, *cc.*	1.0	0.75	0.5	0.25	0.0	1.0	0.0
B. dysenteriæ Shiga resistant, *cc.*	0.0	0.25	0.5	0.75	1.0	0.0	1.0
0.1 cc. Laudman Shiga filtrate diluted	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0
Plates poured and incubated at 37°C. overnight.							
Maximum size of plaques, *mm.*	1	1	1	1	—	Even layer of growth.	
Plaque count	70	100	70	73	No plaques.		
Character of plaques	Clear.	Fairly clear.	Faint.	Faint.			

pose suspensions of susceptible and of resistant bacteria were prepared from respective 18 hour cultures on agar, and an experiment identical in other details with that above was performed.

The presence of resistant bacteria had no effect on either the count or size of the plaques (Protocol 7). However, the plaques appeared to the naked eye less distinct, were faint, as the proportion of resistant bacteria was increased. The pale plaques were altogether indistinguishable under the low power of the microscope from the background of bacterial growth.

Effect of Specificity of Bacterial Substratum on the Size of Plaques.

In dealing with several lytic filtrates of different origin we have observed several instances in which a given filtrate exhibiting activity against two or more species of bacteria produced plaques of different size, depending on the bacterium used. In order to see whether it is the bacterial substratum or the lytic filtrate which is the decisive factor determining the size of plaques, the following experiment was performed.

Laudman Shiga filtrate, which was also active to a less extent on *Bacillus coli*, was increased in its potency for the latter organism by a

Protocol 8.

The Size of Plaques Produced by Certain Lytic Principles on Cultures of Heterologous Bacteria.

	Laudman Shiga filtrate.				*Coli* filtrate.			
	Produced from cultures of Shiga bacillus.		Produced from cultures of colon bacillus.		Produced from cultures of colon bacillus.		Produced from cultures of Shiga bacillus.	
Plated with.....	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*
Maximum size of plaques, *mm*.....	1.2	0.6	1.2	0.6	0.6	0.6	0.6	0.6

series of passages in broth until its titer for colon bacillus reached 10^9 units per cc. Likewise a lytic agent, originally lytic for colon bacillus, was transferred in a series of passages with Shiga bacillus. A sufficient number of transfers was made in each case to eliminate by dilution all traces of the original lytic filtrate. The original Laudman Shiga lysin was then plated in 1 per cent agar with both *Bacillus dysenteriæ* Shiga and *Bacillus coli*. The filtrate resulting from "adaptation" of this lysin to *Bacillus coli* was also plated with both organisms. Similarly, the lytic agent originally active upon colon bacillus, and the product of its "adaptation" to Shiga bacillus, were each plated with both organisms as shown in Protocol 8. The concentration of agar was in all cases 1 per cent.

It appears from the above experiment that the Laudman lytic agent, whether produced in cultures of *Bacillus dysenteriæ* Shiga or in those of *Bacillus coli* retained the characteristic of forming larger plaques with *Bacillus dysenteriæ* Shiga and smaller plaques on plates of *Bacillus coli*. The lytic agent anti-*Bacillus coli* produced a plaque of about 0.6 mm. in diameter when grown with either *Bacillus dysenteriæ* Shiga or *Bacillus coli* irrespective of the culture from which the filtrate was obtained.

The size of the plaque formed on plates of a given bacterium is, then, a function of the lysin, and is retained without change through the process of so called adaptation.

DISCUSSION.

The results of our experiments indicate that the lytic principle acts as though it were present in the medium in the form of indivisible units. While it was suggested by d'Hérelle that these active units are actually organized living particles, the evidence so far produced by him in support of this view does not seem convincing. Since active filtrates consisting of water with a very small percentage of solids, comprising, in addition to active principle, salts, tissue extractives, metabolic products, and bacterial debris, can still be diluted 10^{10} times before reaching the limit of their activity, it is equally possible to suppose that the units of active agent are molecules of some inanimate material.

It is also possible that lytic substance is present in the medium in a state of true solution, and is merely adsorbed on bacterial debris and, being distributed with it, appears as being particulate itself. In such case the number of particles of bacterial debris present will determine indirectly the particulate concentration of active agent. This possibility is especially suggestive if it is remembered that the highest number of active units present in a lytic filtrate at the height of its activity is about 1×10^{10} per cc.—a concentration which very closely approaches the figure expressing the peak of the concentration of susceptible bacteria in a cc. of the medium.

Furthermore, the particles of lytic agent do not behave as if they were living organisms, as shown by the effect on them of comparatively slight changes in concentration of agar. Even as slight an increase

in consistency of the agar as that from 1 per cent to 2.5 per cent suppresses over 99 per cent of plaques. Moreover, it is difficult to reconcile the changes in the size of plaques, as brought about by various procedures in our experiments, with the view of d'Hérelle that the plaques represent the "colonies" of bacteriophage. For instance, if the size of the plaque depends on the progress of "invasion" of bacteria by the bacteriophage why should it be affected by the concentration of agar? Moreover, the more numerous the susceptible bacteria around the initial particle, the more they should be "invaded;" each of these, according to d'Hérelle, in turn should become a source from which the next generation of bacteriophage would invade the nearest bacteria, and in consequence the larger should be the plaque, or at least it should not be smaller. As a matter of fact, however, the greater the concentration of susceptible bacteria, the smaller are the plaques. Similarly it would be difficult to explain, on the basis of d'Hérelle's hypothesis, why both the number and the size of plaques decrease with the increase in proportion of *old* bacteria which are not subject to lysis, whereas the increase in concentration of young *resistant* bacteria leaves both the number and the size of plaques unaffected.

On the other hand, a logical explanation of these phenomena can be given on the basis of the observed tendency of agar and of old bacteria to adsorb the lytic agent, thus interfering with the progress of its diffusion. Since resistant bacteria do not adsorb the lytic principle, other conditions being equal, the presence of an excess of resistant bacteria does not interpose an obstacle for normal diffusion of the lytic agent—hence the difference in their respective effect on the size of the plaques.

SUMMARY AND CONCLUSIONS.

The experiments reported above confirm the fact that lytic principle is distributed in active solution in a state of indivisible units. This permits its quantitative evaluation by serial dilution, as well as by plating on agar. The latter method, however, often gives readings considerably lower than those obtained by the broth dilution method of titration. By varying the concentration of agar it has been possible to show that the discrepancy is due to adsorption of the lytic agent on agar. When the concentration of the latter is increased from 0.3 per

cent to 2.5 per cent the number of plaques of lysis is reduced more than 100 times. At the same time the average size of the plaques also decreases approximately to one-tenth of the original.

The size, as well as the number of plaques, has been found to depend also on the condition of the culture employed in titration. Thus, when the culture exposed to the action of lytic agent is composed of young susceptible bacteria, the greater the concentration of bacteria, the smaller the plaques.

When the culture is composed partly of young and partly of old susceptible bacteria, both the size and the number of the plaques are diminished with the increase in the relative concentration of old bacteria. On the other hand, presence in the culture of resistant bacteria does not affect either the size or the number of the plaques so long as the relative concentration of susceptible bacteria in the culture is sufficient to allow formation of them. The plaques appearing in the presence of a high concentration of resistant variants in the culture are relatively indistinct owing to overgrowth.

Under carefully controlled conditions the size of plaques is found to be determined by the character of the lytic filtrate. Thus in the case of lytic agents which act upon more than one bacterial species the size of the plaques remains constant, irrespective of the bacterial substratum used for the production of the active filtrate.

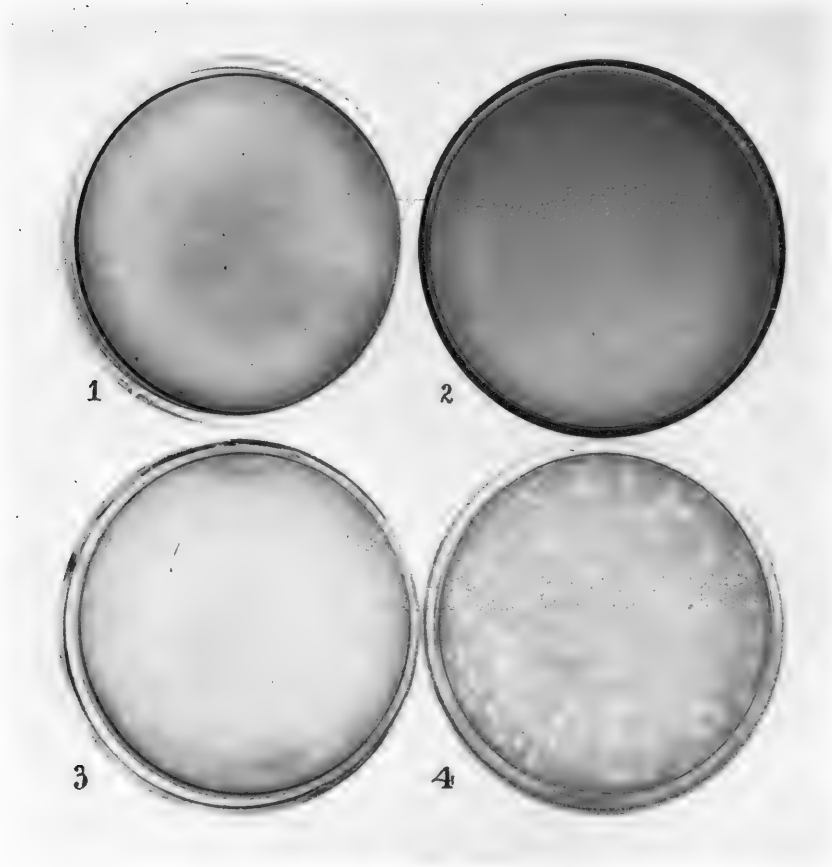
EXPLANATION OF PLATE 20.

FIG. 1. 4.5 per cent agar + 0.1×10^{-1} cc. or $100{,}000 \times 10^{-7}$ cc. of phage—no plaques.

FIG. 2. 2.5 per cent agar + 0.1×10^{-4} cc. or 100×10^{-7} cc. of phage—130 plaques 0.25 mm. in diameter.

FIG. 3. 1 per cent agar + 0.1×10^{-7} cc. of phage—180 plaques 1.0 mm. in diameter.

FIG. 4. 0.5 per cent agar + 0.1×10^{-7} cc. of phage—281 plaques 2.5 mm. in diameter.



(Bronfenbrenner and Korb: Bacteriophage of d'Hérelle.)

EXPERIMENTAL RICKETS IN RABBITS.

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(*From the Department of Pathology of the School of Medicine, Western Reserve University, Cleveland.*)

Plates 21 to 23.

(Received for publication, June 15, 1925.)

INTRODUCTION.

Experimental rickets has been induced by dietetic means in dogs (Mellanby (1)), in rats (McCollum and collaborators (2); Sherman and Pappenheimer (3); and many others), and in swine (Zilva, Golding, and Drummond (4)). Although rickets is said to occur in rabbits (Jost and Koch (5)), up to the present time the experimental production of the disease in this animal has not been reported. One of us (Goldblatt), at the Lister Institute, London, attempted to study the effect of various deficient diets on the growth and on the bone development of rabbits and guinea pigs, but failed because the animals invariably refused to eat the synthetic diets and died.

For studies of metabolism and of blood changes in experimental rickets it is obviously an advantage to be able to produce the disease in a large yet relatively inexpensive laboratory animal. The purpose of the present investigation has been to determine whether or not it is possible to produce the disease in the rabbit by stomach tube feeding of diets which produce rickets in rats.

EXPERIMENTAL.

Series A.

Rabbits from 4 to 6 weeks of age and weighing from 250 to 500 gm. were employed in this investigation. They were kept in individual cages in a room shielded from direct sunlight. Every litter was divided into two groups, one receiving the rickets-producing diet and the other the same diet containing 2 per cent cod

liver oil. In some of the experiments the cod liver oil was administered separately, by pipette, and in others, mixed with the food. Two diets were used.

Diet I.—

Egg albumin (Merck)	20 gm.
Wheat starch	50 "
Cottonseed oil	15 "
Salt mixture (No. 4, Korenchevsky (6))	5 "
$CaCO_3$	2 "
Yeast (Fleischmann's)	5 "
Agar	5 "

Diet II.—This was No. 3143 of McCollum and collaborators (7), modified by the addition of 2 gm. of powdered agar per 100 gm. of the mixture.

Both diets are deficient in the antirachitic organic factor and have a low phosphorus but high calcium content. All of the solid ingredients were finely powdered and the diet mixtures passed through a fine flour sieve. To the ration, just before feeding, were added 2.5 cc. of decitrated lemon juice and an amount of distilled water only sufficient to make the ration fluid enough to pass with ease through a No. 12 catheter which was used as a stomach tube. No difficulty was experienced in the matter of passage of the stomach tube. In this investigation the tube has been passed about 1000 times. In the beginning, 5 gm. of the diet was given twice daily, and at the end of 3 days this was increased to $7\frac{1}{2}$ gm. After 1 week the ration was increased to 10 gm. and at the end of 10 days to 15 gm. twice daily. This quantity was continued to the end of the experimental period.

One complication seriously interfered with the earlier experiments in this investigation. Within a week or two from the time artificial feeding was begun most of the animals, controls included, developed diarrhea, lost weight very rapidly, and died.

Of twelve rabbits that received Diet I, only two survived as long as 3 weeks. Of these two, at autopsy, one had severe rickets and the other moderate rickets. Those that died in 2 weeks or less showed only varying degrees of osteoporosis. Of eight control animals on Diet I containing 2 per cent cod liver oil, one lived as long as $3\frac{1}{2}$ weeks. None of the controls showed any signs of rickets; but the bones were osteoporotic.

Only three out of twelve rabbits that received Diet II lived as long as 3 weeks and of these one had severe rickets and the other two moderate rickets. The remaining nine had merely varying degrees of osteoporosis. Seven control rabbits that received Diet II containing 2 per cent cod liver oil lived for varying periods up to $3\frac{1}{2}$ weeks. All

finally developed severe diarrhea and died but showed no macroscopic or microscopic evidence of rickets.

The intestinal disturbance did not appear to be of infectious origin. At autopsy there was no evidence of acute enteritis or colitis, but the entire small and large intestine contained feces in a liquid or semi-solid state. There seemed to be a total inability to concentrate the intestinal contents.

Series B.

In order to supply the animals with more roughage than they were getting from the agar in the diets, advantage was taken of the work of Nelson and Lamb (8), who advised the administration of alfalfa to rabbits kept on synthetic diets. They used alfalfa meal, but we used whole alfalfa hay devitaminized by extraction for 36 hours with boiling alcohol in a flask with reflux condenser attached, and then by still extraction for 3 or more hours with ether. 5 gm. of the dried extracted alfalfa was allowed to every animal daily. They ate it readily, and from the time the administration of alfalfa began diarrhea ceased to develop.

Diet I.

Of a litter of five rabbits (see Table I) three (Nos. 3, 4, and 5) were given Diet I and alfalfa and two control rabbits (Nos. 1 and 2) received Diet I containing 2 per cent cod liver oil and the same ration of alfalfa. For 2 weeks they were fed Diet I by stomach tube and then it was found by trial that these rabbits ate the diet voluntarily. For the remaining 2½ weeks the rabbits were allowed to eat of their own accord and consumed on an average 35 gm. of the diet and 5 gm. of alfalfa daily.

X-Ray Examination.—At the beginning of the experiment, and at intervals of 1 week thereafter, radiograms of the animals were made. In the three rabbits on Diet I and alfalfa, definite abnormalities of the skeleton suggestive of developing rickets were noticeable by x-ray as early as 1 week after the commencement of the experiment, and from week to week the radiograms showed progressive changes. In the radiograms taken immediately before the animals were killed the rachitic metaphysis was very wide and long, there was definite cupping and fuzziness of the end of the diaphysis and imperfect calcification of the epiphysis. In the radiogram of one of the controls (No. 1; Fig. 2)

TABLE I.

Rabbit No.	Sex.	Diet.	Weight.		Inorganic phosphate in blood serum.	Calcium in blood serum.	Diagnosis.		
			Initial.	Final.			X-ray.	Macroscopic.	Microscopic.
			gm.	*gm.*	*mg. per cent*	*mg. per cent*			
1	F.	No. I + cod liver oil.	456	620	8.9	11.9	No rickets.	No rickets.	No rickets.
2	"	" I + " " "	469	640	8.17	10.5	" "	" "	" "
3	"	" I	505	775	3.25	13.5	Severe rickets.	Severe rickets.	Severe rickets.
4	"	" I	455	715	3.1	13.0	" "	" "	" "
5	"	" I	395	660	3.1	11.55	" "	" "	" "

TABLE II.

Rabbit No.	Diet.	Weight.		Inorganic phosphate in blood serum.	Calcium in blood serum.	Diagnosis.	
		Initial.	Final.			Macroscopic.	Microscopic.
		gm.	*gm.*	*mg. per cent*	*mg. per cent*		
6	No. II + cod liver oil.	357	625	8.6	13.2	No rickets.	No rickets.
7	" II + " " "	327	550	6.1	12.5	" "	" "
8	" II + " " "	325	Died early.				
9	" II	380	620	3.8	13.7	Moderate rickets.	Moderate rickets.
10	" II	385	Died early.			Osteoporosis.	Osteoporosis.
11	" II	365	595	3.8	13.08	Moderate rickets.	Moderate rickets.
12	" II	340	380	3.6	10.85	Slight rickets.	Slight rickets.

a slight irregularity appeared at the end of the 3rd week, in the zone of provisional calcification, which persisted to the end. Control Rabbit 2 remained normal throughout the experimental period.

Clinical Examination.—In Rabbits 3, 4, and 5, deformity of the chest and enlargement of the costochondral junctions and of the epiphyses were apparent at the end of 2 weeks, and at the end of the experimental period these changes were very pronounced. Pot belly and muscular weakness developed during the last 10 days.

Macroscopic Examination.—At autopsy all the signs of very severe rickets were present in Rabbits 3, 4, and 5, and there was no gross evidence of rickets in the two control animals.

Microscopic Examination.—Histologically, the diagnosis of very severe rickets in Rabbits 3, 4, and 5 was confirmed, while the controls (Nos. 1 and 2) showed only slightly deficient osteogenesis in the slender trabeculæ of the primary spongiosa, but these were numerous and well calcified and there was no other abnormality (see Fig. 6).

Chemical Examination.—From every rabbit, at the end of the experimental period, about 25 cc. of blood was obtained from the carotid artery. The amount of calcium in the blood serum was determined by the Kramer-Tisdall (9) method, and the amount of inorganic phosphate by the Tisdall method (10). In the rachitic animals the calcium content of the serum was slightly greater than in the controls, but the inorganic phosphate of the serum was very much lower (see Table I).

Diet II.

A litter of seven rabbits was given Diet II and extracted alfalfa. Three rabbits were chosen as controls and received the same diet containing 2 per cent cod liver oil. One of the controls died 3 days after the beginning of the experiment but the cause was not determined at autopsy. One animal on Diet II died of bronchopneumonia 11 days after the commencement of feeding. All were fed by stomach tube for 3 days and from then on allowed to eat of their own accord. They consumed on an average 30 gm. of the diet and 5 gm. of extracted alfalfa daily. The litter were killed at the end of 2½ weeks. Grossly and histologically, the controls showed no evidence of rickets while the remaining three rabbits showed from slight to moderate rickets. Chemical examination showed only slight differences in the percentage of calcium in the blood serum of the rachitic and control rabbits but a great diminution in the amount of inorganic phosphate in the blood serum of the rachitic rabbits (see Table II).

SUMMARY.

Rickets has been produced experimentally in rabbits by the administration of diets deficient in the antirachitic organic factor and phosphorus but with a high calcium content.

Cod liver oil added to these rickets-producing diets prevented the development of rickets in rabbits.

We are indebted to Professor H. T. Karsner for constant encouragement and valuable criticism. It is with great pleasure that we acknowledge sincere thanks to Professor T. W. Todd whose deep interest in the work and continued aid greatly facilitated the investigation, and to Miss W. Kuenzel for valuable technical assistance in the radiographic work. All the radiograms were taken in the Department of Anatomy.

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EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. A series of x-ray photographs of part of the right fore limb of Rabbit 3 that received the rickets-producing Diet I and extracted alfalfa. (*a*) to (*e*) were taken at intervals of 1 week, (*f*) was taken 3 days after (*e*). Note the progressive separation between epiphysis and diaphysis of radius and ulna caused by the development of the rachitic metaphysis, the cupping and imperfect calcification of the end of the diaphysis, and the imperfect calcification of the epiphysis.

FIG. 2. A series of x-ray photographs of part of the right fore limb of Control Rabbit 1 that received Diet I containing 2 per cent cod liver oil, and extracted alfalfa. The photographs were taken at the same time as those represented in Fig. 1. At the end of the 3rd week (*d*) a slight irregularity appeared at the junction of epiphysis and diaphysis of the ulna and this persisted. Histologically, there was no evidence of rickets in this zone, but the hypertrophic zone of proliferating cartilage showed a localized increase in the number of layers of cells. The zone of provisional calcification was very densely calcified.

FIG. 3. X-ray photographs of part of right hind limb of Rabbit 3 showing progressive changes at the epiphysеodiaphyseal junction in the lower end of the femur and the upper end of the tibia, similar to those described for the radius and ulna of the same rabbit (see Fig. 1).

FIG. 4. Right hind limb of Control Rabbit 1. Normal throughout the experimental period.

PLATE 22.

FIG. 5. Rabbit 3. Photomicrograph of part of the lower end of the radius. Section stained with hematoxylin and eosin. Note the great number of layers of hypertrophic cartilage cells in the zone of proliferating cartilage, and the partial disorganization of this zone by the ingrowth of blood vessels and bone marrow. The zone of provisional calcification is absent. The trabeculæ of the primary spongiosa have a slender densely calcified core which is surrounded by a relatively large amount of osteoid tissue; and the cortical bone also shows excessive subperiosteal as well as endosteal osteoid. Diagnosis: severe rickets.

FIG. 6. Control Rabbit 1. Photomicrograph of part of lower end of radius. Section stained with hematoxylin and eosin. The trabeculæ of the primary spongiosa are short and slender but numerous and very well calcified. The zone of provisional calcification is well calcified. There are only from four to six layers of hypertrophic cartilage cells in the zone of proliferating cartilage. Diagnosis: slight osteoporosis.

PLATE 23.

FIG. 7. Rabbit 3. Photomicrograph of costochondral junction. Section stained with hematoxylin and eosin. Note the great deformity; the excessive proliferation of hypertrophic cartilage cells; the absence of a zone of provisional calcification; and the excessive amount of osteoid tissue in the region of the primary spongiosa as well as in the cortex. Diagnosis: severe rickets.

FIG. 8. Control Rabbit 1. Photomicrograph of costochondral junction. Section stained with hematoxylin and eosin. The trabeculæ of the primary spongiosa are slender but well calcified. There is a definite zone of provisional calcification. The zone of hypertrophic cartilage cells consists of only four layers. The cortical bone is thin and rarified, but what is left is well calcified. There is less than the normal amount of osteoid surrounding the trabeculæ and in the cortex. Diagnosis: moderate osteoporosis.

a b c d e f

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

a b c d e f

(Goldblatt and Moritz: Experimental rickets in rabbits.)

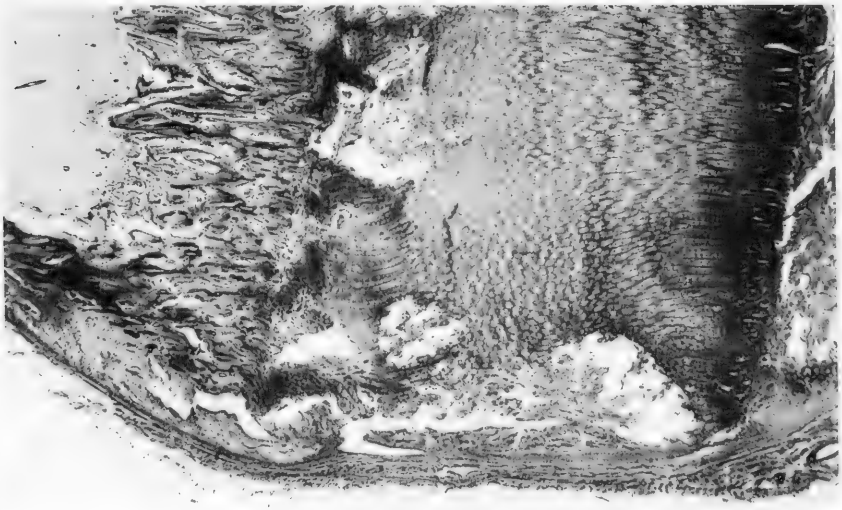

FIG. 5.

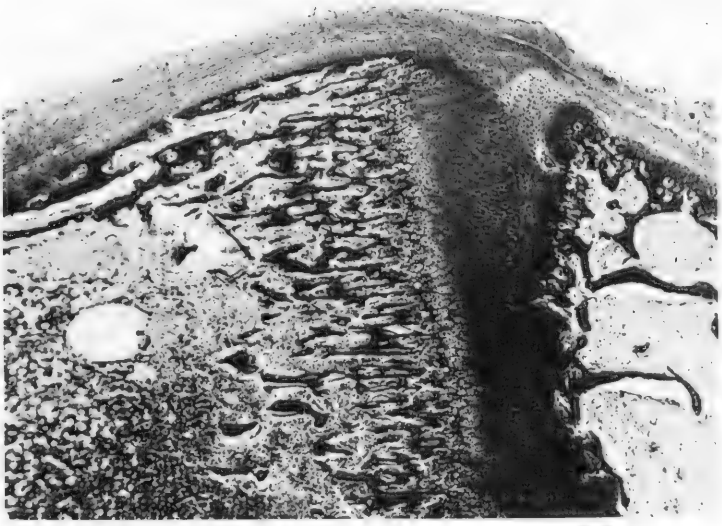

FIG. 6.

(Goldblatt and Moritz: Experimental rickets in rabbits.)

FIG. 7.

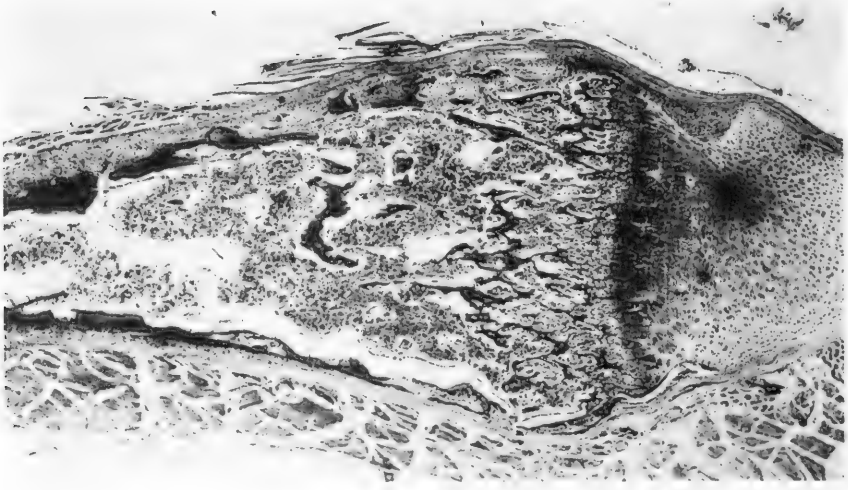

FIG. 8.

(Goldblatt and Moritz: Experimental rickets in rabbits.)

STUDIES ON REGENERATION OF BACTERIOPHAGE.

I. The Influence of Partial Anaerobiosis upon Regeneration of a Highly Diluted Lytic Principle.

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(Received for publication, June 22, 1925.)

INTRODUCTION.

Quantitative studies on the potency of bacteriophage have established that 1/100,000,000 cc. per cc. of broth or a dilution 10^{-8} of this principle is usually the smallest amount which is able to produce an appreciable lysis. The dilutions are made in a series of tubes containing 4.5 cc. of broth. If larger volumes of broth are used a dilution of bacteriophage still greater than 10^{-8} is able to produce an almost complete lysis. This last observation was made by Gratia and De Kruif (1).

Doerr and Zdansky (2) corroborated Gratia and De Kruif. They pointed out that although it is probable that a certain minimal absolute quantity of lytic principle is necessary for regeneration, as claimed by Gratia and De Kruif, this assumption must be made with some reserve. They also drew attention to the possibility that for a given high dilution 1000, 100, and 10 cc. still contain a few elements of the bacteriophage, while 1 cc. may have none. As is seen these authors did not reach any definite conclusion about the nature of the phenomenon under discussion.

The authors mentioned apparently paid no attention to the possibility that the conditions for the regeneration of a highly diluted bacteriophage may not be identical in the various containers employed on account of differences in the supply of air. Although Gratia and De Kruif did not state the capacity of the containers used it is assumed by the writer that the tubes were those of standard size usually employed for broth cultures in bacteriological laboratories and that

1000 cc. of fluid was put in the "2 liter" Erlenmeyer flasks. In the latter case the access of oxygen[1] to 1000 cc. of fluid in a "2 liter" Erlenmeyer flask and to 10 cc. of broth in a 120 × 22 mm. tube is correspondingly 35 and 10 times more restricted than to 1 cc. of broth in a 120 × 22 mm. tube.

Preliminary experiments have shown that the degree of exposure to air of a highly diluted bacteriophage plays an important rôle in the regeneration of a principle so diluted. In view of this observation the following investigations were planned:

1. To determine whether the results obtained by Gratia and De Kruif depend upon differences in the access of air to lytic cultures contained in vessels of various sizes.

2. To study the relation of partial anaerobiosis to the regeneration of a highly diluted lytic principle.

Methods.

The media employed for this work were always adjusted to pH 7.6 and prepared from Difco meat extract.

The bacteriophage kindly sent to me by Dr. J. Bronfenbrenner was obtained by him from feces of a convalescent from Shiga dysentery by employing d'Hérelle's technique. This principle was then adapted to *B. coli* in this laboratory.

The strength of this phage, as well as that of the ones regenerated under various conditions, was always tested by titration in broth (Appelmans (4)) and its quantitative value expressed by Werthemann's lytic exponents recorded symbolically as E_L (5) (the dilution of the last tube showing lysis).

The count of plaques, as proposed by D'Hérelle for quantitative estimation of the potency of bacteriophage, was never attempted since it had been pointed out by Bronfenbrenner (6) that the number and appearance of these areas depend on many factors, such as concentration of agar, moisture of the surface of the slant, etc., which may considerably interfere with the reliability of results obtained.

Since the degree of lysis and the titer of the bacteriophage may sometimes depend to a certain degree on the size of the *inoculum* a uniform inoculum was used throughout the experiments so far as possible. Unless otherwise stated, about 400,000 bacteria per cc. were usually inoculated. The emulsion was always prepared by washing a 24 hour agar slant culture of *B. coli* and diluting it with normal salt solution to the required density.

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[1] The access of oxygen to a fluid can be measured by the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio (Avery and Morgan (3)).

EXPERIMENTAL.

I.

In repeating Gratia and De Kruif's experiment with the anticolon bacteriophage employed in this laboratory, the following results were obtained for a 10^{-9} dilution of this principle in broth inoculated with 20 million *Bacillus coli* per cc.

1000 cc. – Erlenmeyer flask of "2 liter" capacity—complete lysis.
100 " – " " " 250 cc. " " "
10 " –120 × 22 mm. test-tube—almost complete lysis.
5 " –120 × 22 " " —no lysis.
1 " –120 × 22 " " " "

According to Gratia and De Kruif's explanation of these results, 1 to 5 cc. are the "non-lytic" absolute quantities. Any amount above 5 cc. contains a "lytic" absolute quantity; *i.e.*, an absolute amount sufficiently large to produce lysis.

These preliminary observations were utilized in the following experiments which were planned to determine whether variations in the absolute quantity of an extremely diluted bacteriophage bear a relation to its regeneration.

A. Complete Regeneration of a Small "Non-Lytic" Quantity of Highly Diluted Bacteriophage under Conditions of Restricted Supply of Air.

The anticolon bacteriophage was diluted up to 10^{-9} in 1012 cc. of broth, inoculated with approximately 20 million *Bacillus coli* per cc.[2] The mixture was shaken and distributed into different containers so that various $\frac{\text{Surface area}}{\text{Total volume}}$ ratios were obtained for "non-lytic" absolute quantities of this principle (1 and 5 cc.). In 24 hours of incubation the degree of lysis was compared macroscopically, then samples from each container were heated to 58° for $\frac{1}{2}$ hour and titrated in broth. Table I represents a summary of results obtained.

As is seen from this table small "non-lytic" absolute quantities (1 and 5 cc.) of 10^{-9} dilution of bacteriophage may produce complete

[2] An inoculum of a similar size was used by Gratia and De Kruif.

lysis and regenerate a lytic principle of the same strength manifested by a large absolute amount, provided that the access of oxygen is restricted to the same degree in both cases.

TABLE I.

Regeneration of a Small, "Non-Lytic" Quantity of Highly Diluted Bacteriophage under Restricted Supply of Air.

Capacity of the container.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
	cc.			
Erlenmeyer flask, 2 liter.....	1000	0.1	+	8
Tube 120 × 8 mm.....	5	0.1	+	8
" 120 × 22 ".....	5	0.7	0	0
" 120 × 22 ".....	1	3.5	0	0
" 120 × 8 "	1	0.5	+	8

+ = lysis; 0 = normal growth.

TABLE II.

Absence of Regeneration of a Large, "Lytic" Amount of Highly Diluted Phage under Conditions of Free Access of Air.

Capacity of the container.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
	cc.			
Erlenmeyer flask, 2 liter.....	100	1.5	0	0
120 × 22 mm. tube.....	2.34	1.5	0	0
120 × 8 " "	5	0.1	+	8
Erlenmeyer flask, 250 cc.....	100	0.1	+	8

+ = lysis; 0 = normal growth.

B. Absence of Regeneration of a Large, "Lytic" Amount of Highly Diluted Bacteriophage under Conditions of Free Access of Air.

This experiment was made in the same manner as Experiment A, there being a difference, however, in the size of containers and amounts of fluid introduced. Table II presents the results obtained.

As is seen, the regeneration of a large volume of highly diluted bacteriophage depends entirely on the restriction in supply of air usually obtained in such volumes. However, if the supply of air becomes

less restricted, there is no regeneration obtained even in a large volume.

II.

These observations on the influence of restriction in supply of air upon the regeneration of highly diluted bacteriophage have been further supported by a study of this phenomenon in relation to different factors, as in the following four experiments.

TABLE III.

The Relation of $\frac{Surface\ Area}{Total\ Volume}$ Ratio to Regeneration of a Highly Diluted Lytic Principle.

Size of tube.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
mm.	*cc.*			
120 × 22	1	3.5	0	0
120 × 22	2	1.75	0	0
120 × 22	3	1.16	0	0
120 × 22	4	0.88	0	0
120 × 22	5	0.7	0	0
120 × 8	1	0.5	+	8
120 × 8	2.5	0.25	+	8
120 × 8	5	0.1	+	8

In the column Lysis, 0 = normal growth; + = lysis.

1. Relation of Various $\frac{Surface\ Area}{Total\ Volume}$ Ratios to Regeneration of a Highly Diluted Bacteriophage.

To determine the restriction of the supply of air necessary for regeneration of a highly diluted bacteriophage, a 10^{-9} dilution of this principle in broth was inoculated with approximately 20 million *Bacillus coli* per cc. Decreasing amounts of this mixture from 5 to 1 cc. were distributed in 120 × 22 mm. and 120 × 8 mm. tubes so that a wide range of $\frac{\text{Surface area}}{\text{Total volume}}$ ratios was obtained. After 24 hours of incubation, readings of the degree of lysis were made macroscopically and

samples from each of the tubes, heated to 58° for ½ hour, were titrated to determine the lytic exponents. Table III gives the details of this experiment and the results obtained.

As will be seen, the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.5 supplies the minimal degree of anaerobiosis required for regeneration of the highly diluted bacteriophage. An increase in the ratio above this point completely prevents the regeneration of bacteriophage.

2. The Rate of Regeneration of Highly Diluted Bacteriophage under Conditions of Partial Anaerobiosis, and the Relation of This Regeneration to the Rate of Bacterial Growth.

To investigate the rate of regeneration of bacteriophage under a restricted supply of air and to establish the relation of this regeneration to bacterial growth, it was necessary to make a quantitative study. This was done as follows:

Bacteriophage was diluted up to 10^{-9} in 10 cc. of broth previously inoculated with *B. coli*. 5 cc. of this mixture was then placed into two tubes measuring 120 × 22 mm. and 120 × 8 mm. respectively with result that $\frac{\text{Surface area}}{\text{Total volume}}$ ratios of 0.7 and 0.1 were obtained. Before incubation and every 30 minutes during the following 6½ hours of incubation, samples were taken from these tubes for counts of viable organisms. At the same intervals of time 0.1 cc. was taken from each tube, added to 9.9 cc. of broth, and heated at 58° for ½ hour. These mixtures were later titrated in broth for determination of their lytic exponents.

Charts 1 and 2 represent the results obtained.

As is seen, the regeneration of a highly diluted lytic principle occurs only under conditions of partial anaerobiosis, begins distinctly at the 3rd hour of incubation, and is completed in about 6 hours. When the growth curves of Charts 1 and 2 are compared, very little difference can be noted in the rate of growth of *Bacillus coli* during the first 3 to 4 hours of incubation. It is suggested, therefore, that partial anaerobiosis does not favor the regeneration of bacteriophage by modifying the rate of bacterial growth, but has some direct relation to the regeneration of this principle. Further investigation, however, is necessary to establish the point definitely.

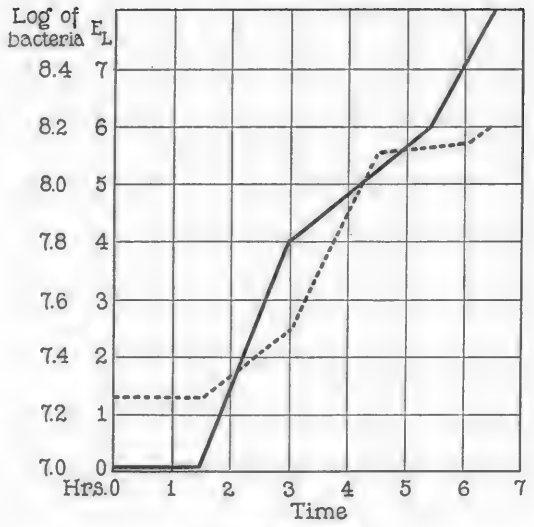

CHART 1.

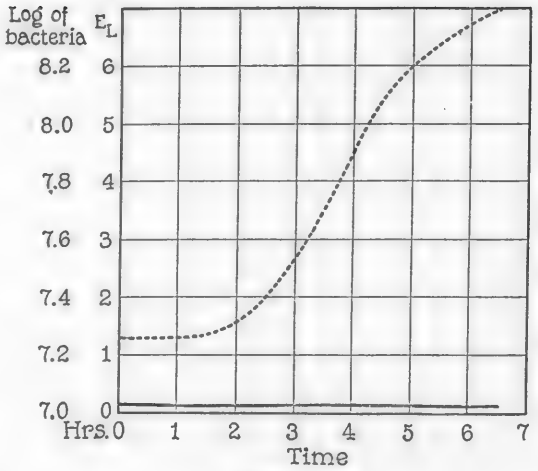

CHART 2.

CHART 1. The relation of regeneration of bacteriophage under partial anaerobiosis to the bacterial growth curve. The dotted line represents the rate of bacterial growth at $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1. The solid line represents the regeneration of bacteriophage at this ratio.

CHART 2. Absence of regeneration of a highly diluted bacteriophage at $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.7. The dotted line represents the bacterial growth at the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.7. The solid line represents the regeneration of a highly diluted phage at this ratio. Through an error in drawing this chart, the curve of bacterial growth was made in free hand.

3. Minimal Time of Restricted Air Supply Required for Regeneration of Highly Diluted Bacteriophage.

The object of this experiment was to find out the shortest time during which the culture with a highly diluted bacteriophage must be kept under conditions of partial anaerobiosis to obtain regeneration.

A 10^{-9} dilution of lytic principle in broth was inoculated with approximately 20 millions *B. coli* per cc. and distributed in 5 cc. quantities into series of 120 × 8 mm. tubes $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.1\right)$ and into one control tube 120 × 22 mm. in size $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.7\right)$. These tubes were placed in the incubator and kept there for various periods of time from 10 minutes up to 2 hours. Every 10 minutes one 120 × 8 mm. tube was taken from the incubator and its contents poured into a 120 × 22 mm. tube which was further incubated up to 24 hours. In this manner a series of cultures kept under the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1 for different intervals of time was obtained. The degree of lysis in these tubes in 24 hours was estimated macroscopically and their lytic exponents determined by titration in broth.

It was found that 1 hour of incubation at the ratio 0.1 was sufficient for complete lysis and full regeneration ($E_L = 8$) of a highly diluted bacteriophage.

4. The Influence of Partial Anaerobiosis upon Regeneration of Highly Diluted Bacteriophage at Different Points on the Bacterial Growth Curve.

In the previous experiment, as was seen, the supply of air restricted to a certain degree for 1 hour at the beginning of the growth curve was sufficient for regeneration of a highly diluted bacteriophage. The object of this experiment was to find out whether any regeneration of such a principle would occur if the conditions of partial anaerobiosis were created at phases of growth following the 1st hour.

In order to investigate this point 5 cc. of *Bacillus coli* cultures containing a 10^{-9} dilution of the bacteriophage was incubated in 120 × 22 mm. tubes $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.7\right)$ for various intervals of time

and then transferred into 120 × 8 mm. tubes $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1) and incubated for 1 hour. At the end of this hour the fluid was poured back into 120 × 22 mm. tubes and incubated to bring the total time of incubation up to 24 hours. A series of counts of viable organisms in each tube was made every 30 minutes for a period of 6 hours. After 24 hours of incubation the degree of lysis in all the tubes was determined macroscopically and their lytic exponents found by titration in broth. Since the counts showed that the growth of bacteria was not interfered with by transferring the culture from 120 ×

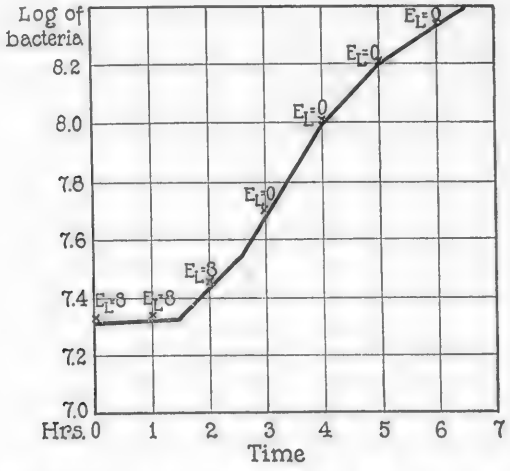

CHART 3. The influence of partial anaerobiosis upon regeneration of a highly diluted bacteriophage at different points of the growth curve.

22 mm. into 120 × 8 mm. tubes for 1 hour, only one growth curve is presented in Chart 3. Crosses at different parts of this curve indicate the points at which the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1 was created for 1 hour. The lytic exponent of the bacteriophage regenerated under corresponding conditions is placed above each cross.

As will be seen from the chart, if one is to obtain regeneration of a highly diluted lytic principle it is necessary to restrict the access of air at the 1st, 2nd, or 3rd hour of growth. At the later phases of

growth no regeneration of a principle so diluted occurs even under restriction of the supply of air.

SUMMARY.

The regeneration of an extreme dilution of bacteriophage in a large volume of broth, as described by Gratia and De Kruif, is due to the influence of partial anaerobiosis upon the regeneration of the principle.

The influence of partial anaerobiosis upon regeneration of an extremely diluted bacteriophage was investigated in relation to different factors with the following results.

1. The $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.5 expresses the degree of anaerobiosis necessary for regeneration of an extreme dilution of the bacteriophage. An increase in this ratio interferes with regeneration.

2. The regeneration of the highly diluted bacteriophage under partial anaerobiosis begins definitely at the 3rd hour of bacterial growth and is completed after about the 6th hour.

3. To obtain the full regeneration of an extreme dilution of bacteriophage it is sufficient to restrict for one hour the supply of air to a culture containing it, providing that this restriction be produced within the first 3 hours of the bacterial growth.

I am indebted to Dr. Louis Gross for helpful suggestions.

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THE PRODUCTION OF PERSISTENT ALOPECIA IN RABBITS BY X-RAYS OF VARIOUS DEGREES OF HARDNESS.

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These studies are a continuation of work reported recently[1] on the minimum dose of soft x-ray required to produce persistent alopecia of the abdominal skin of white rabbits. For the purposes of that work rays produced at 30 kilovolts, peak, were employed, and the critical dose was found to correspond to the production in air of 2.04×10^{15} pairs of ions per gm. The rabbits were found to react accurately; although eighteen of the exposures fell within 2 per cent of the critical value, half on either side, no single case of inconsistent reaction occurred.

The present paper deals with similar experiments on the effect of somewhat harder rays. The various factors which have to do with the quality and intensity of the radiation are given in Table I. Column 1 shows the peak kilovoltage at which the rays were produced; Column 2, the current in milliamperes; Column 3 the thickness of the aluminium filter in mm.; and Column 4, the target distance in cm.

Apparatus.

The same x-ray plant has been used in this work as in the previous work. The plant is known to operate with a high degree of consistency. The experiments were made at various times over a period of about 1 year, during which time four different tubes were used, and minor adjustments of the electrical equipment were made. As a means of following the tube efficiency and the effect of adjustment, the rate of ionization of air by the rays was measured from time to time. The apparatus used to make these measurements, together with studies of its trustworthiness, has been discussed.[2] The measurements were made with the ioniza-

[1] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

[2] Clark, H., *Am. J. Roentgenol. and Radium Therap.*, 1924, xi, 445.

tion chamber in place of the exposed portion of the animal. A necessary correction for the effect of scattered radiation has been included in all values used in this paper. It varies from 2 per cent at 40 kilovolts, to 4 per cent at 70 kilovolts. The method of determining it has been described.[1]

Preparation and Treatment of the Animals.

Only normal healthy white rabbits weighing between 1500 and 2100 gm. were used. The method of preparing and exposing them to the radiation has been described in detail.[1] Before exposure, the abdomen was shaved and covered with a screen of lead-filled rubber in which there are seven apertures, six round, 1 cm. in diameter, and one elongated for the purpose of identification. During exposure, the animal was shielded carefully from the heat of the tube and kept cool by a current of air at room temperature.

TABLE I.

1	2	3	4
40	15	0.5	27
50	12	1.0	27
70	8	1.5	36

RESULTS.

In order to avoid the possibility of bias in making measurements and diagnoses, the work has been divided into two parts from the beginning. One of us (Sturm) has attended to the preparation and exposure of the animals, and to the observation of them thereafter, while the dosage measurements were made by the other (Clark). The two sets of records were not compared until each had been put into final form.

Fig. 1 is the record of the alopecia reactions of the animals. It comprises all of the experiments made at 40 and 50 kilovolts except a few preliminary ones in which the dose given proved to be too small to yield results of interest. The results at 70 kilvolts are not shown since in no case was the exposure sufficient to produce persistent alopecia. Each vertical column of circles gives the record of one rabbit, the seven circles corresponding to the seven skin areas treated. Each circle is so plotted on the vertical scale as to show the corresponding length of exposure in minutes. White circles represent temporary alopecia, the figures within showing the time interval after exposure, in weeks, before the regrowth of hair was observed. Black circles repre-

sent alopecia persistent for at least 10 weeks. In a few cases, a very slight regrowth was first observed at 9 weeks. In no case, however, was a similar observation made at 10 weeks, although most of the animals were kept under observation for some time thereafter. Reference to Fig. 1 shows that with one exception,—in which the regrowth was observed at 7 weeks,—all of the lowest black circles of each group lie on the same horizontal line, although the figures in the highest white circles are variable; five of the six 9's in the figure correspond to doses within 3 per cent of the critical dose as calculated below. It is assumed, therefore, that observations made at 10 weeks or thereafter constitute a fair criterion of persistency. Nine groups of animals are represented in Fig. 1—six at 40 kilovolts and three at 50 kilovolts. The four animals of each group were exposed on the same day to rays from the same x-ray tube.

Table II contains a summary of the ionization measurements together with all of the data from Fig. 1 which are of interest for the purpose of determining the critical dose. In addition to the work at 40 and 50 kilovolts, Table II contains the measurements and data relative to the longest exposures at 70 kilovolts. Column 1 contains the identification numbers of the various groups of animals for purposes of reference to Fig. 1. Columns 2 and 3 show the degree of hardness of the rays (kilovolts), and the number of the tube used, respectively. Column 4 contains the summary of the ionizing powers of the rays when the various tubes were used; the figures given are the numbers of pairs of ions (divided by 10^{14}) produced per gm. of air per minute at the surface of the abdomen of the animal. The critical exposure time for each group of animals dealt with in Fig. 1, lies somewhere between the times which correspond to the highest white circles and the lowest black circles; Columns 5, 6, and 7 of Table II have to do with these highest white circles, and Columns 8, 9, and 10, with the lowest black ones. Of these, Columns 5 and 8 contain the number of circles considered; Nos. 6 and 9 contain the exposure times taken from Fig. 1; and Nos. 7 and 10 contain the products (divided by 10^{15}) obtained by multiplying the exposure times of Columns 6 and 9 respectively by the ionization rates of Column 4.

We may make use of Columns 7 and 10 to estimate the critical dose for each kind of ray, in ionization terms. The critical dose must not

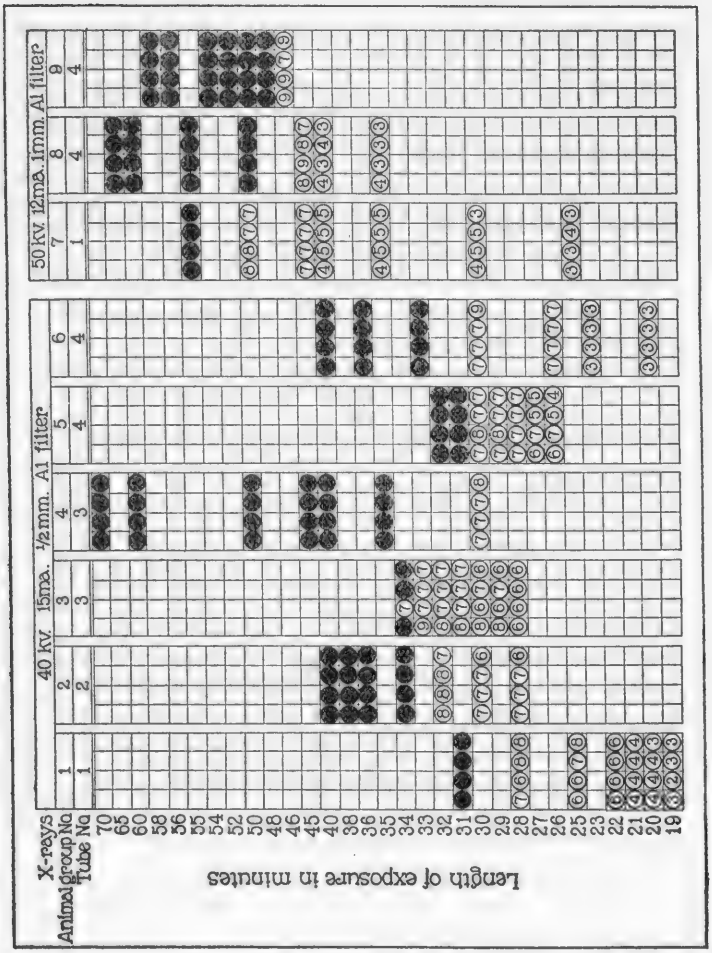
X-rays
Animal group No.
Tube No.
40 Kv. 15ma. ½mm. Al filter
50 kv. 12ma. 1mm. Al filter
Length of exposure in minutes

FIG. 1.

be less than any of the values given in Column 7 nor greater than any of the values in Column 10. At 40 kilovolts no such value appears, either because the measurements are inaccurate, or because the animals do not react consistently, or because the output of x-ray is not constant. The discrepancy is not great, however; if we may assume that the value given in Column 7 for one animal (*), is too high by 2/3 of 1 per cent, the table becomes consistent for the critical value 3.68×10^{15}. This value is then defined by 19 black circles, and 9 white circles, all of which lie within 2.5 per cent of it. Since the crit-

TABLE II.

1	2	3	4	5	6	7	8	9	10
1	40	1	1.19	4	28	3.33	4	31	3.69
2	40	2	1.10	4	32	3.52	4	34	3.74
3	40	2	1.10	1*	34	3.74	3	34	3.74
4	40	3	1.05	4	30	3.15	4	35	3.68
5	40	4	1.20	4	30	3.60	4	31	3.72
6	40	4	1.20	4	30	3.60	4	33	3.96
7	50	1	0.90	4	50	4.50	4	55	4.95
8	50	4	0.955	4	45	4.30	4	50	4.78
9	50	4	0.955	4	46	4.39	4	48	4.58
	70	1	0.49	4	80	3.92			
	70	3	0.51	4	100	5.10			

ical value, as written, implies a higher degree of accuracy than can be claimed for the measurements, it may be taken as 3.7×10^{15} pairs of ions per gm. At 50 kilovolts, the table is consistent; the critical dose may be taken as 4.5×10^{15}. At 70 kilovolts, the critical dose is at least greater than 5.10×10^{15}.

To sum up the foregoing, the critical dose is 3.7×10^{15} pairs of ions per gm. at 40 kilovolts, 4.5×10^{15} at 50 kilovolts, and over 5.10×10^{15} at 70 kilovolts.

DISCUSSION.

These experiments, taken with the earlier ones,[1] show that, as regards the alopecia reaction, rabbits respond, under constant conditions of treatment, with little or no individual variation. They show also that when rays of different degrees of hardness are considered, the

critical dose, expressed in terms of ionization, is not constant; at 30, 40, 50, and 70 kilovolts the critical doses are 2.04, 3.7, 4.5, and over 5.1 respectively (all multiplied by 10^{15} pairs of ions per gm.). It is of interest to note also that the alopecia, erythema, and pigmentation reactions do not run parallel to each other. At 30 kilovolts, the critical alopecia dose produced little erythema—none in most cases. At higher voltages, it produced progressively more pronounced erythema. At 30, 40, and 50 kilovolts, it produced no pigmentation, but at 70 kilovolts, one-half of the animals receiving doses above 3.05×10^{15}, which is, of course, far below the critical dose, became pigmented.

There is no evident explanation of these results. The dosage values given refer to the surface of the animal. Hard rays produce more ionization within the body of the animal than soft rays, for the same surface ionization; and to produce the same surface dosage, the exposures are necessarily longer. It seems quite clear that x-rays produce primary changes in matter only through the medium of ionization produced by secondary beta rays, and that the amount of ionization produced is proportional to the energy absorbed irrespective of the hardness of the ray. It seems clear also that the relation between the ionization of the skin tissues and that of air is within reasonable limits independent of the quality of the ray. The complex effects of x-rays on animals probably result from simple primary reactions.

SUMMARY.

1. The minimum dose of x-ray, of each of three degrees of hardness, required to produce persistent alopecia of the abdominal skin of normal white rabbits has been studied. 40 kilovolt rays filtered by 0.5 mm., 50 kilovolt rays filtered by 1 mm., and 70 kilovolt rays filtered by 1.5 mm. of aluminium were employed. The 70 kilovolt work is unfinished.

2. Under constant conditions of treatment, the animals were found to react with a high degree of consistency.

3. The values of the critical minimum doses expressed in terms of air ionization (pair of ions per gm. of air divided by 10^{15}), for rays produced at 30, 40, 50, and 70 kilovolt peak, are 2.04, 3.7, 4.5, and over 5.1 respectively. The 30 kilovolt value is taken from a previous paper.[1]

4. It is shown that the alopecia reaction does not run parallel to the erythema and pigmentation reactions.

GROWTH AND PERSISTENCE OF FILTERABLE VIRUSES IN A TRANSPLANTABLE RABBIT NEOPLASM.

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(Received for publication, June 18, 1925.)

Observations concerning the growth and persistence in a transplantable rabbit tumor of two filterable viruses, Virus III and vaccine virus, are reported in this paper.

In 1923 Levaditi and Nicolau (1) reported observations concerning the growth and survival of vaccine virus in neoplasms of mice and rats. Evidence favoring his theories of dermotropism and neurotropism of viruses was considered by him to be found in the fact that vaccine virus does not grow or survive in mouse or rat sarcoma but does multiply in mouse cancer and survives throughout the life of the neoplasm. Among other conclusions he stated that cancer cells are unable to develop an active immunity against infection with vaccine virus and only passively partake of their host's immunity.

Levaditi's observations and conclusions are interesting and, if confirmed, are of importance in regard to the growth of viruses in general and, also, in regard to the biology of tumor cells, particularly their immunological response to infection. A unique opportunity for the study of certain problems of this nature is afforded by our possession of a virus and of a transplantable neoplasm of the same animal species—the rabbit. In beginning this work it was decided to determine, first, if the virus would grow in the tumor, and if so what effect this condition would have upon the character of the malignant disease. The present paper is concerned primarily with the first of these questions.

Methods and Materials.

Virus III.—While attempting to produce chicken-pox in rabbits a filterable transmissible pathogenic agent was discovered (2). This agent produces gross as well as microscopic lesions in the cornea, skin, and testicles of rabbits, and an

infection with it leads to an immunity against subsequent infections with the same material. For convenience this agent has been spoken of as Virus III. The virus has been transferred from rabbit to rabbit at 3 to 5 day intervals over a period of 2½ years. It acts like a fixed virus in that a small amount of it when injected into the testicles of rabbits causes a sharp rise of temperature to 104–106°F. 3 to 5 days after the inoculation, and because intradermal injections of the virus in dilutions of 1:1000 are regularly followed by visible skin reactions. Although the virus can be recovered from the heart's blood of rabbits following intratesticular or intradermal inoculations, it does not cause death even when injected intracerebrally. The testicular emulsions containing the virus have been shown repeatedly to be free from ordinary anaerobic and aerobic bacteria by means of cultures on blood agar, in broth, and in Smith-Noguchi tubes. Furthermore, ordinary bacteria have not been seen in stained films and dark-field preparations of the emulsions containing the virus, in stained sections of inoculated testicles, or in sections of inoculated testicles impregnated with silver nitrate. Local infiltration of the tissues with endothelial leucocytes, swelling of the involved epithelial cells, and the presence of nuclear inclusions in both the endothelial leucocytes and the epithelial cells are the characteristic pathological changes observed in rabbits experimentally inoculated with the virus. At first it was considered not unlikely that the virus is the etiological agent of varicella. Further work, however, in our laboratory and also in Swift's (3), disclosed the fact that Virus III is indigenous to rabbits. From the work completed, it seems most likely that Virus III is as typical a virus as vaccine virus or the virus of symptomatic herpes from both of which it can easily be differentiated.

In the experiments to be reported, the presence or absence in rabbits of an immunity to Virus III was determined by means of intradermal inoculation of the virus or by testing the virucidal properties of the sera.

Vaccine Virus.—The vaccine virus used in these experiments was obtained from Noguchi (4) and is a strain which has been propagated during a number of years by frequent passage from rabbit to rabbit by testicular inoculation. Inoculation of the scarified skin or cornea of rabbits is followed by typical macroscopic and microscopic lesions. The presence or absence in rabbits of an immunity to the virus was determined by intradermal inoculation of it.

Neoplasm.—A number of papers dealing with the tumor employed in these experiments have already been published (5). Suffice it to say that the tumor arose in the scrotal skin of a rabbit which eventually showed marked constitutional symptoms and finally succumbed with a widespread distribution of metastatic growths. Successful transplantation from the original animal through successive generations was accomplished by intratesticular inoculations (6). The original growth appeared to be of epithelial origin, composed of cells allied to those found in the bulb and root sheath of the hair. The general morphology of the cells and the structure of the growth have been unaffected by transplantation.

Although various routes of inoculation may be used with which a primary growth and, at times, metastases are obtained, it has been found that intratesticular

inoculation is most satisfactory from the point of view of an actively developing and persistent primary tumor and for the study of the disease process as a whole. As a routine procedure, a group of not less than ten rabbits is inoculated each month from an actively growing primary tumor of the previous month's series. In the present paper tumors from such groups are referred to as "stock tumors". The general character of the disease in different groups fluctuates from time to time, and, furthermore, there are well defined differences in the disease picture of individual animals in any single group. Such factors as the growth and fate of the primary tumor, the animal incidence of metastases, their number, extent, and distribution, and the actual and probable mortality rates are significant variables in this connection. However, for the purpose of the present paper, it is sufficient to say that an actively progressing primary tumor is obtained by intratesticular inoculation in practically every instance and that by the 14th day after inoculation the growth has frequently replaced the entire testicle. In a certain number of rabbits the primary tumor continues to grow, involving the cord and extending into the abdominal cavity; in others regression, absorption, and eventual healing occur, while some animals continue to carry a large primary tumor composed of both living and necrotic tissue for many weeks.

From an analysis of the postmortem examinations of the first twenty generations of the tumor (7), the mean incidence of secondary growths for the entire group of animals was 60 per cent and upon the same basis the average mortality varied from 20 to 40 per cent. Metastases have been found in practically every organ and tissue of the body, the most common sites being the kidneys, lungs, suprarenals, liver, eyes, and the serous membranes of the abdomen. There occur, also, cord and retroperitoneal tissue extensions from the primary growth. Gross metastases have been found as early as 2 weeks after inoculation and there is no doubt that emboli of tumor cells are distributed soon after inoculation of the testicle. Death from extensive and widely distributed tumor growth has occurred as early as 3 weeks after inoculation and in some instances actively growing metastases were still present in animals killed as late as 7 months after inoculation. On the other hand, some rabbits whose primary tumor attains large proportions are able completely to suppress and eventually to heal this growth and at autopsy no signs of secondary tumors are to be found.

The characteristic feature of variation in the disease, as shown by individual animals of the same group and by different groups of animals, has been considered highly significant from the point of view of animal reaction and animal economy. It should be emphasized that in any study of the effect which various agents, such as the filterable viruses, may have upon the growth and malignancy of the tumor, one must take into account this biological characteristic of the disease. This phase of our study is reserved for a further report.

EXPERIMENTAL.

Growth and Survival of Virus III in the Tumor.

A number of young actively growing tumors were inoculated with Virus III to determine, first of all, if the virus would grow and persist in the tumor. Details of these experiments will be omitted since early in the work we were confronted with the fact that all the stock tumor rabbits, into which no virus had been intentionally inoculated, became immune to infection with Virus III. Table I summarizes a few of the experiments showing the development of an immunity in the stock tumor rabbits to Virus III. A comparable number of animals inoculated with *Treponema pallidum* or *Treponema pertenue* and housed under conditions similar to those of the tumor rabbits were used as controls.

The immunity of the stock tumor rabbits to Virus III gains in significance when it is realized that the stock tumor is transferred only at monthly intervals, and that all of the animals develop immunity to the virus 2 to 3 weeks after inoculation with the tumor. Several possible explanations for this refractory state occurred to us; namely, (1) that the virus, accidently introduced into the tumor, multiplies and survives longer in it than in testicular tissue and was being transferred each month with the tumor, (2) that the tumor at one time accidently infected with the virus had itself acquired an immunity which it conferred upon all its subsequent hosts, or (3) that the tumor in some non-specific manner protected its host against infection with the virus.

In proceeding to investigate this question, attempts were first made to demonstrate the presence of Virus III in the stock tumor by means of rapid passage of emulsions of the primary tumor or metastases through several series with not more than three successive rabbits in each. The tumor tissue selected for these experiments appeared, on gross examination, to be living for the most part. It will become obvious further on in the report why more than three passages in each series were not made. By means of the procedure it was possible to demonstrate regularly the presence of Virus III not only in the primary tumor but also in metastases. 54 days was the longest interval elapsing between the intratesticular inoculation of the tumor and the

TABLE I.

Summary of Experiments Showing that the Inoculation of Rabbits with Stock Tumor Was Followed by a Refractory State of the Animals to Virus III.

Experiment No.	No. of rabbits.	Test for immunity to Virus III before inoculation.	Inoculation.	Site of inoculation.	Interval between inoculation and test for immunity to Virus III.	Method of testing for immunity to Virus III.	Result.
					days		
I	5	Not made.	Tumor.	Skin.	24	Intradermal inoculation of Virus III.	All immune.
II	5	" "	"	"	49	" "	" "
III	8	" "	"	Right testicle.	61	" "	" "
IV	3	" "	"	" "	45	Virucidal properties of sera.	All virucidal.
V	5	" "	"	" "	14	" "	" "
VI	10	None of the sera virucidal for Virus III.	"	" "	25	" "	" "
					mos.		
VII	20	Not made.	Syphilis or yaws.	" "	3–12	Intradermal inoculation of Virus III.	None immune.

The action of Virus III was controlled in each instance either in the skin of normal and immune rabbits or by virucidal tests with sera from normal and immune rabbits.

test for the presence of the virus in the primary growth and metastases. In Text-fig. 1 some of the experiments which demonstrate the presence of Virus III in the primary and metastatic growths are graphically illustrated.

Although inoculation of stock tumors, 4 to 8 weeks old, into the testicle led to an immunity in rabbits to Virus III, no visible virus reaction was obtained by means of intracutaneous inoculations of the same material. From previous work with Virus III it has been shown that amounts of virus too small to give visible skin reactions are sufficient to immunize the animals and that these small amounts of virus are sufficiently increased after two or three testicular passages to produce visible reactions. From the experiments thus far reported, one may conclude that Virus III is regularly present in stock tumors, 4 to 8 weeks old, in quantities sufficient to immunize rabbits but not great enough to produce visible skin reactions. As we were interested not only in the presence but also in the survival of the virus in the neoplasm, it was essential that we work with older tumors. Nevertheless, we were also interested in determining if intracutaneous inoculations of young stock tumors would produce visible Virus III reactions. A number of stock tumors, 7 to 10 days old, were removed from testicles or skin, emulsified, and tested under properly controlled conditions for their ability to produce visible Virus III reactions in the skin of normal rabbits. It was found that intracutaneous inoculations of such emulsions were followed by typical virus reactions (Text-fig. 1). In this connection it is of interest and of probable significance to note that nuclear inclusions, typical of Virus III reactions, were found in the young stock tumors. Similar inclusions, on the contrary, have not been seen in older tumors nor in a strain of the tumor free from the virus which will be described later in the paper.

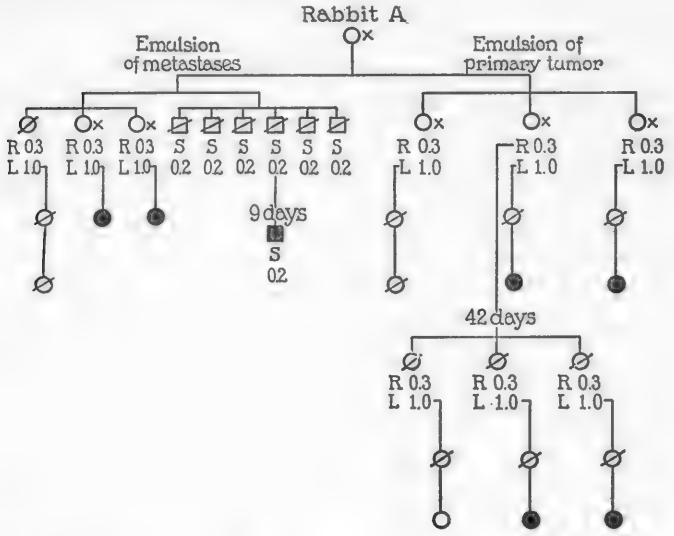
The results of the work thus far show that Virus III is present in the stock tumor and is being transferred with it each month, that it multiplies in the tumor and survives there for at least 54 days. Since it has been shown that rabbits inoculated with the stock tumor themselves became immune to Virus III 2 or 3 weeks later, the survival of the virus in the tumor for 8 weeks evidently takes place despite the immune state of the rabbit host.

Text-Fig. 1.

Growth and Survival of Virus III in a Transplantable Rabbit Tumor.

Oct. 15, 1924, right testicle of Rabbit A inoculated with 0.3 cc. stock tumor emulsion. Tumor grew and metastasized.

Dec. 8, 1924, *54 days later*, rabbit sacrificed and portions of primary tumor and metastases in retroperitoneal, axillary, mesenteric, and mediastinal lymph glands were injected into stock rabbits as indicated below.



Each circle represents one rabbit.

R indicates right testicle; L, left testicle; S, skin. Figures following R, L, and S indicate amounts in cc.

Passages from left testicles to testicles of subsequent animals were made at 4 day intervals. Further passages from rabbit to rabbit were made at 4 day intervals by means of bilateral testicular inoculations of testicular emulsions. Intradermal inoculations were made in the majority of the rabbits. Passage from R was made from tumor in right testicle.

● indicates that a visible Virus III lesion was noted in the skin, that the testicles contained a virus which was identified as Virus III, and that the animal was immune 3 weeks later to Virus III inoculated intradermally.

ø indicates absence of gross virus lesions; rabbit immune to Virus III 3 weeks later.

○ indicates absence of gross virus lesions; rabbit not immune to Virus III 3 weeks later.

× indicates that immunity of the rabbit to Virus III was not tested.

▱ indicates a female rabbit inoculated intradermally with tumor emulsion and immune to Virus III 3 weeks later.

▪ indicates a visible Virus III lesion in skin of female rabbit produced by emulsion of tumor removed from skin of another female rabbit. Test controlled in the skin of a rabbit immune to Virus III.

Growth and Disappearance of Virus III Injected into the Testicles of Normal Rabbits.

It seemed that 4 to 8 weeks is an unusually long time for Virus III to survive in the tumor. From previous work it had been found that Virus III injected into the testicles of normal rabbits multiplies rapidly, probably reaching the maximum growth between the 4th and 6th days. The virus also disappears rapidly from the testicles and frequently no visible reaction is obtained in the skin with emulsions of testicles removed 8 days after inoculation. The mere fact that no visible reaction is obtained is not adequate proof that all the virus is dead, and, in view of the long survival of the virus in the tumor, it seemed essential to investigate its length of survival in the previously normal animal after intratesticular inoculation. Consequently, the following experiments were performed to determine when one can be reasonably certain that the virus is no longer alive in the experimentally infected rabbit.

Rabbit 1 received 1.0 cc. of a testicular emulsion containing active Virus III in each testicle and 0.2 cc. in the skin. The usual virus reaction occurred in the skin and testicles. 34 days later the animal was etherized and both testicles and the inguinal, popliteal, and axillary lymph glands were removed, emulsified, and injected into the testicles of three normal rabbits. A month later these rabbits were tested for the presence of an immunity to Virus III by means of intracutaneous inoculations of the virus and all were found to be susceptible.

Rabbit 2 was inoculated in a manner similar to Rabbit 1. Both testicles were taken out under ether anesthesia 4 days later. 30 days after inoculation the inguinal, axillary, and popliteal lymph glands were removed, emulsified, and injected into the testicles of two normal rabbits. A month later these rabbits were tested for the presence of an immunity to Virus III and were found to be susceptible.

The above experiments afford indirect evidence that Virus III is no longer present in the testicles and lymph glands of the experimentally infected rabbit a month after the inoculation or, if present, that it exists in such small quantities or in such condition as to be incapable of inducing immunity. It seemed advisable to pursue the matter further by means of rapid passage of the emulsions of lymph glands and testicles from experimentally infected animals through several series of not more than three successive stock rabbits in each to obtain,

if possible, some visible or immunological evidence concerning the survival of the virus. A month was the period of time arbitrarily chosen to elapse between the inoculations and the attempts to demonstrate a viable virus in the experimentally infected rabbits. Three experiments were conducted as outlined and after the passages had been made all the animals were held for 3 or 4 weeks, after which time they were tested for the presence of an immunity to Virus III.

It must be remembered in connection with these experiments that Virus III is indigenous to rabbits and that it was first discovered by the serial transfer, at 3 or 4 day intervals, of rabbit's testicles which in the first instance had been inoculated with blood from chicken-pox patients. In the work on varicella, macroscopic evidence of Virus III was noticed only once in twelve series earlier than the fourth passage. If the passages were continued further, macroscopic reactions became numerous for the reason that each passage increased the chances of encountering a rabbit spontaneously infected with the virus and of concentrating the virus sufficiently to produce visible reactions. In the light of this experience, it is obvious why not more that three successive rabbits were employed in a series in the experiments reported in the present paper. In connection with the tests for the presence of an immunity in the rabbits held 3 or 4 weeks, as mentioned above, it must also be remembered that a certain number of stock rabbits are immune to Virus III. Since the virus is indigenous to rabbits, this condition is to be expected. The percentage of immune animals from miscellaneous stocks and from a number of different sources has been variable, but a fair average is 15 to 20 per cent.

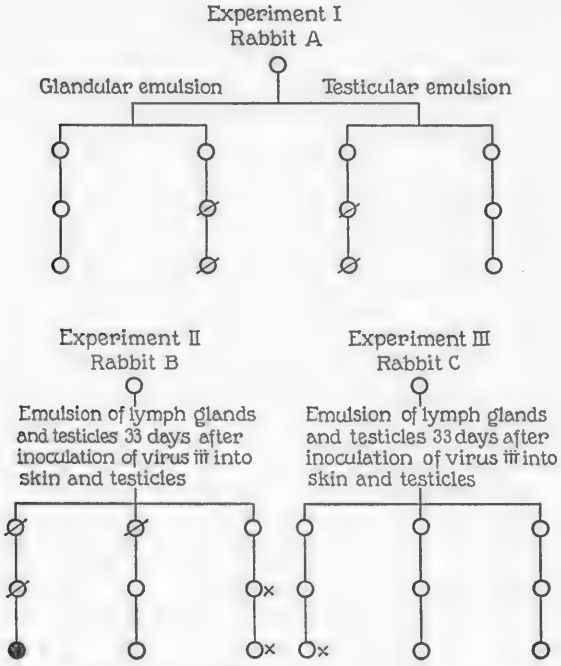
Examination of the results of the experiments graphically illustrated in Text-fig. 2 reveals that only one visible Virus III skin reaction was obtained in ten series and that only 20 per cent of the first rabbits to be inoculated in the ten series were immune to Virus III 3 weeks after inoculation. Since these results coincide with those obtained in previous work in which the series were begun with human blood, one may be reasonably certain that Virus III is not present in the testicles and lymph glands of experimentally infected rabbits a month after inoculation. A comparison of the results of the experiments with the stock tumor (Text-fig. 1) with those shown in Text-fig. 2 reveals that Virus III survives much longer in the tumor than it

Text-Fig. 2.

Absence of Virus III in Lymph Glands and Testicles of Rabbits 1 Month after Inoculation.

Nov. 1, 1924, skin and testicles of Rabbit A were inoculated with Virus III. Marked reaction in skin and testicles.

Dec. 4, 1924, *33 days later*, both testicles and axillary, popliteal, and inguinal glands removed, emulsified, and injected into testicles of stock rabbits as indicated below.


Experiment I
Rabbit A
Glandular emulsion
Testicular emulsion
Experiment II
Rabbit B
Emulsion of lymph glands and testicles 33 days after inoculation of virus III into skin and testicles
Experiment III
Rabbit C
Emulsion of lymph glands and testicles 33 days after inoculation of virus III into skin and testicles

Each circle represents one rabbit.

Passage from animal to animal was made by means of testicular emulsions injected into testicles of stock rabbits at intervals of 4 days. The skin was also inoculated in each rabbit.

● indicates that a visible Virus III lesion was noted in the skin, that the testicles contained a virus which was identified as Virus III, and that the rabbit was immune 3 weeks later to Virus III inoculated intradermally.

ø indicates absence of gross virus lesions. Rabbit immune to Virus III 3 weeks later.

○ indicates absence of gross virus lesions and that the rabbit was not immune to Virus III 3 weeks later.

× indicates that immunity of the rabbit to Virus III was not tested.

does in testicular tissue. The reasons for the unusually long survival of the virus in the tumor are being investigated at present.

Strain of Tumor Free of Virus III.

It is not known when the tumor became infected with Virus III. After it was found that the tumor carries the virus, the question naturally arose as to whether the character of the tumor is affected by its presence. Previous work has shown that the injection of Virus III alone does not produce tumors. It was not known, however, whether the presence of the virus affects the growth of the tumor cells in such a way as to influence the malignancy of the disease. To determine what effect the virus has upon the tumor it was essential to obtain a strain of the tumor free of the virus. The work with the stock tumor, bearing the virus, led us to believe that this would be difficult of accomplishment inasmuch as the virus survives in the tumor many weeks after the rabbit host becomes refractory to skin infection with the virus and after its blood serum is highly virucidal. Nevertheless, an accident enabled us to obtain a strain of the tumor entirely free of Virus III.

A rabbit inoculated with the stock tumor bearing the virus died several weeks later and accidently lay in a warm room 12 to 18 hours. The primary tumor was removed and inoculated into the testicles of three rabbits. It was fully 3 weeks before one could be sure that the transplants were increasing in size. Growth, once initiated, progressed, however, and transfers were successfully made from one of the tumors. A number of generations of this strain of the tumor have been studied and we are convinced that Virus III is no longer present in it inasmuch as it has been impossible to demonstrate its presence (Text-fig. 3) by methods which proved successful with the stock tumor. Moreover, animals inoculated with this strain do not become refractory to skin infection with Virus III and their sera do not become virucidal (compare Text-figs. 1 and 3). In addition, no nuclear inclusions, typical of Virus III reactions, have been found in young virus-free tumors.

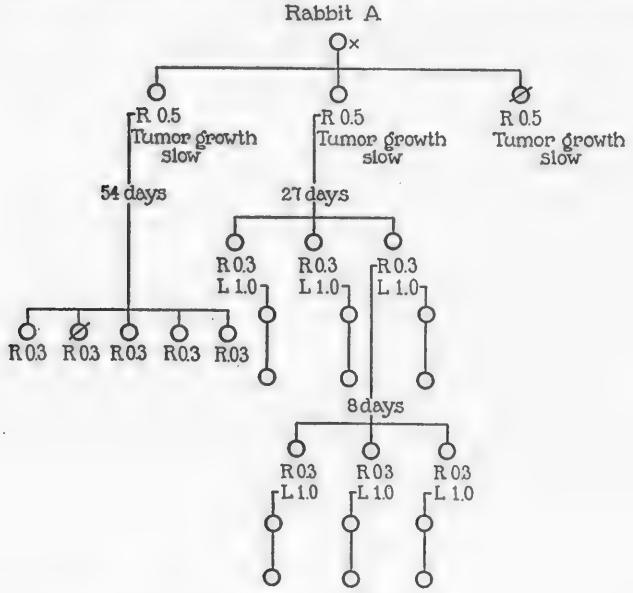
These findings show conclusively that the virus is not essential for the growth of the tumor inasmuch as the strain of tumor free of Virus III grows, metastasizes, and causes death. Further than this one cannot go at present. The behavior, however, of the tumor bearing the virus in comparison with that of the tumor without the virus is being studied.

Text-Fig. 3.

Strain of Transplantable Rabbit Tumor Free of Virus III (a Virus-Free Strain of Transplantable Rabbit Tumor).

Oct. 15, 1924, right testicle of Rabbit A inoculated with 0.3 cc. of same stock tumor emulsion used in inoculating Rabbit A in Text-fig. 1. Tumor grew.

Nov. 13, 1924, rabbit died and lay in a warm room 12 to 18 hours. Primary tumor removed, emulsified, and inoculated into stock rabbits as indicated below.



Procedure and symbols same as those in Text-fig. 1.

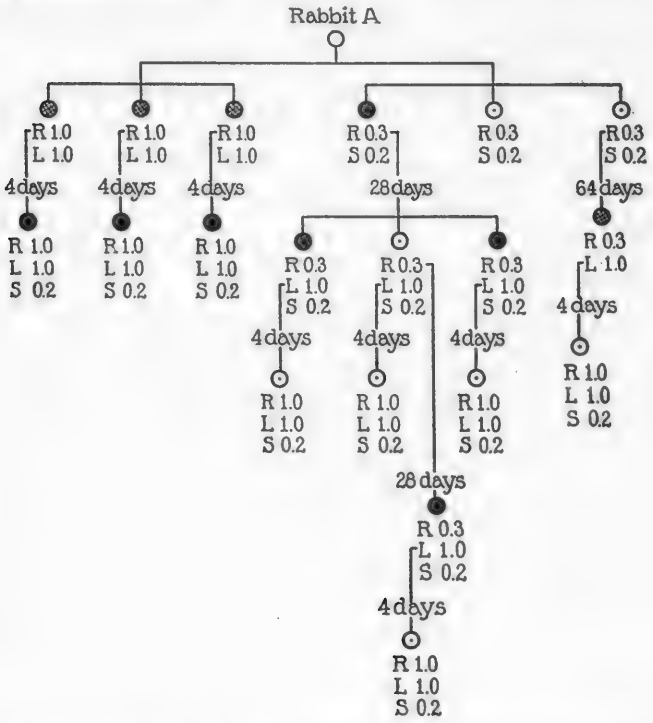
Growth and Survival of Vaccine Virus in the Tumor.

When it was found that Virus III grows in the rabbit tumor and survives there for an unusually long time, it seemed probable that confirmatory or additional facts in regard to the problem under study might be obtained by similar experiments with vaccine virus. Therefore, a young tumor was inoculated with vaccine virus, removed 4 weeks later, and passed to several normal rabbits by means of intratesticular inoculations. The tumor was experimentally infected only

Text-Fig. 4.

Growth and Survival of Vaccine Virus in a Transplantable Rabbit Tumor.

Oct. 15, 1924, right testicle of Rabbit A inoculated with tumor. Growth.
Oct. 31, 1924, tumor inoculated with vaccine virus. Continued tumor growth.
Nov. 28, 1924, *4 weeks later*, tumor removed, emulsified, and inoculated into rabbits as indicated below.



Each circle represents one rabbit.

R indicates right testicle; L, left testicle; S, skin. Figures following R, L, and S indicate amounts in cc.

Transplants of tumor were made in right testicles at intervals of 28 to 64 days as indicated.

Passages from animal to animal for the demonstration and identification of vaccine virus in the tumor were made as indicated at 4 day intervals by injecting tumor or testicular emulsion into the testicles and skin of rabbits.

● indicates presence of vaccine virus in testicles. Animal immune to vaccine virus inoculated intradermally 3 weeks later.

⊙ indicates presence of vaccine virus in testicles, vaccine pustules in skin. Animals not tested for immunity.

● indicates presence of vaccine virus in testicles, vaccine pustules in skin. Animals immune to vaccine virus inoculated intradermally 3 weeks later.

the one time. Several generations of this strain of tumor have been studied, transfers being made at intervals of 28 to 64 days. All rabbits inoculated with it become refractory to skin infection with vaccine virus and the presence of vaccine virus in the tumor has been repeatedly demonstrated. A few of the experiments are graphically illustrated in Text-fig. 4.

Noguchi (4) recovered vaccine virus from experimentally infected testicles of the rabbit 28 days after inoculation. He was unable to recover it later than this and places the outside limit of survival at 5 weeks. We have been able to demonstrate the virus in tumors several generations removed from the experimentally infected tumor and it is evident that it multiplies and is carried to successive generations of the tumor. Since the tumor transplants at times were made at intervals of 64 days it is also apparent that vaccine virus survives longer in the tumor than it does in testicular tissue, despite the fact of the immune state of the rabbit host. This condition is similar to that encountered in connection with Virus III.

DISCUSSION.

The experiments reported in this paper confirm Levaditi's observations concerning the growth and unusually long survival of vaccine virus in neoplasms. In addition, we have shown that a filterable virus indigenous to rabbits behaves in a similar manner in a transplantable neoplasm of the same animal species. We agree with him that malignant cells may not be able to develop an active immunity to viruses and that this may be the explanation for the long survival of the viruses in the rabbit tumor of our experiments. We feel, however, that a great deal more evidence is needed before this explanation can be accepted as the correct one. If it be correct, there will remain the further question whether the inability of cells to develop an active immunity is unique with tumor cells. In any event, it would seem that the use of a transplantable actively growing tissue, such as the rabbit tumor, offers a suitable medium *in vivo* and possibly *in vitro*, for the growth and study of a number of viruses and possibly of other agents of disease. Such procedures may also lead to observations which will have a significant bearing upon the biological reaction to various infectious agents of tumor cells as contrasted with normal cells.

SUMMARY.

Virus III and vaccine virus multiply in a transplantable rabbit tumor of epithelial origin; are carried along with the tumor through an indefinite number of transplants; and despite an immunity developed by the rabbit host survive longer in the tumor than when injected into the testicles of normal rabbits.

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EXPERIMENTAL STUDIES WITH A SPIRAL ORGANISM FOUND IN A WILD RAT AND IDENTICAL WITH THE ORGANISM CAUSING RAT-BITE FEVER.

Second Paper.

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Plates 24 and 25.

(Received for publication, November 5, 1924.)

In an earlier paper I reported the findings in white rats and guinea pigs experimentally infected with a spiral organism found in a wild rat, and expressed the opinion that the organism is identical with *Spirochæta morsus-muris*. The experiments yielded an interesting difference in the course of the infection in white rats and guinea pigs. Whereas the rats showed only a transient blood infection without clinical signs of illness or tissue involvement,—except for slight keratitis and conjunctivitis—all guinea pigs succumbed to the infection, and showed chronic blood infection and marked tissue involvement. Conjunctivitis, keratitis, and marked infiltration of the eyelids and the soft parts of the nose were found to be very constant symptoms. The present paper deals with the pathology of rabbits infected with the spiral organism which has been preserved by passages through guinea pigs since October, 1922.

The passages have been performed by subcutaneous injection of a single drop of blood of an infected guinea pig into two or three healthy guinea pigs. Occasionally secretion from the eyes was used for transmission. This proved successful in every instance and, as above stated, all guinea pigs succumbed to the infection, showing well developed blood and tissue involvement. I emphasize the fact because guinea pigs injected with material taken from infected rabbits failed in some instances to show clinical signs of illness, and while spirochetes could not be demonstrated in their blood, they harbored the spirochetes in the tissues. This latent infection in guinea pigs will be dealt with in the course of the present paper. It is noteworthy that in the long series of transmission experiments from guinea pig to guinea pig the injection was followed after 3 to 7 days by a more

or less pronounced local reaction in the form of a fairly circumscribed edema and subsequent induration in nearly all of the animals. The few animals which failed to develop a primary lesion had been inoculated with secretion from the eyes, in which the direct microscopic examination often did not reveal any spirochetes, or with blood from animals recently dead. The transmission with the blood of these dead animals I have used lately for economic reasons, as this method induces a very chronic infection in the guinea pigs with especially pronounced tissue involvement. Animals so infected live 2 months or even longer, as a rule, whereas guinea pigs injected with blood taken at the height of blood infection generally die before the end of the 5th week.

Experiments with Rabbits.

Two series of animals were used in these experiments. The first series was comprised of rabbits which were injected under aseptic precautions subcutaneously or intracorneally with blood of guinea pigs which on microscopical examination showed numerous spirochetes. The second series was of non-infected animals which were mated with animals of the first series.

All rabbits were in excellent general condition without any lesions of the skin or mucocutaneous borders. The temperature was taken daily for a week before the experiment and was found to be within the normal limits.

Series 1.

This comprised ten rabbits, five males and five females. Five of the animals were injected subcutaneously at the base of the right ear, two into the scrotum or vulva, one in the back, and two were inoculated intracorneally. The amount injected subcutaneously was 0.1 to 0.5 cc. The inoculation into the cornea was performed with a sterile capillary glass tube. The amount so injected was about 0.03 cc. All animals were kept separated after inoculation and examined daily.

1. Primary Lesion.

The injection at the base of the ear was followed in all animals after 3 to 5 days by an ill defined local edema and intense reddening of the skin. The vessels were greatly injected and the ear felt distinctly hotter than the normal one. In one animal edema and inflammation were only moderate and subsided almost completely within 3 days. In the others it spread in a few days around the whole base of the ear. Two animals showed small blebs filled with clear fluid over the center of the swelling where the inoculation had been made. The doughy inflammatory edema soon became indurated, and falling of the hair began with eventual complete loss of it. This was accompanied by desquamation of the epidermis and

superficial ulceration covered by adherent dry crusts. The duration of the primary lesion could not be determined accurately as swelling and infiltration of the bases of the ears appeared in the 3rd and 4th weeks after inoculation in nearly all animals, as a symptom of systemic infection. The animal which was injected in the back developed a similar edema followed by marked induration of the whole thickness of the skin with subsequent falling out of hair and formation of dry adherent scabs. This lesion persisted for 2 months, healing slowly, first in the center, leaving a dry rough skin completely devoid of hair. The reaction of the animals inoculated into the soft skin of the genital region was a much stronger one. Intense injection and edema appeared in both on the 6th day after inoculation. 2 days later the summit of the swelling was covered with small blebs containing clear fluid. The center of the lesion was now much indurated and was surrounded by a pronounced, transparent, non-inflammatory edema which covered nearly the whole scrotum or vulva. One animal developed a deep necrosis of the cutis, whereas in the other only an irregular necrotization of the epidermis took place. Here again it was not possible to draw conclusions as to the duration of the primary lesion, as swellings, induration, and subsequent ulceration of the genital region in males and females was found to be a very constant sign of the general distribution of the spirochetes in the rabbit. The two rabbits inoculated into the cornea developed an interstitial keratitis between the 6th and the 7th days. In one animal this keratitis was only slight, consisting of a milky cloudiness in the upper segment of the cornea where the inoculation was done. It disappeared entirely within a week, leaving no signs other than a slight episcleral injection and edema along the rectus superior muscle. In the other animal the reaction was very pronounced. There was intense cloudiness of a great part of the cornea accompanied by pronounced episcleral injection and edema, and fine vessels entered from the periphery far into the cornea. Also in this part of the body the ultimate course of the primary lesion could not be studied accurately as marked involvement of the eyelids and conjunctivæ proved to be an early and constant sign of general infection. The rapidly moving spirochetes, with their typical bipolar flagella, could always be demonstrated in serum aspirated from the primary lesions of the skin but they were never found in as large numbers as in the primary lesions of guinea pigs. In the secretion of the eyes of the animals inoculated into the cornea I found them only after long and repeated search. As soon as the edematous swelling of the skin was replaced by a hard induration, the spirochetes could no longer be demonstrated by direct microscopical examination in the aspirated serum.

2. General Infection.

For 10 to 13 days after inoculation the rabbits did not show any conspicuous signs of illness, and there was no fever. After this second period of incubation, however, pronounced involvement of the skin

over the head and genital regions appeared suddenly, and led in a few days to marked alteration of the parts involved. The constancy and regularity with which these signs of general infection set in were very striking.

The first sign in all animals was a puffiness of the face, especially around the eyes. This puffiness disappeared quickly and a more or less marked edema of the upper eyelids appeared. The edema soon became infiltrated and inflamed. The conjunctivæ, especially of the upper lids, became injected, and there was discharge from the eyes, at first mucoid but soon becoming mucopurulent. The lower lid became involved also, but rarely to such an extent as the upper one. The eyelashes, at first glued together by the mucopurulent discharge, fell out more or less completely. There appeared in most animals superficial ulcerations of the upper eyelids covered by dry crusts. The hair around the eyes began to fall out as in the guinea pigs inoculated with this organism. In a few animals a slight keratitis developed in the upper segment of the cornea. In some animals involvement of the genital region appeared at the same time as the infiltrations of the eyelids, but in the majority this appeared a few days later. It consisted of an edematous swelling of the prepuce and the scrota in males and of the entire vulva in females. The anus was involved also but generally less intensely. In two males the edema of the scrota was very pronounced. At the beginning it was transparent and soft but soon became inflamed and much infiltrated. In three males the testicles became swollen suddenly overnight; this swelling increased rapidly and the testicles felt quite hard. The skin over them was tense and shiny and superficial cracks appeared. In a few days the epidermis of the scrotum became dark brown, at some places nearly black, dry, and hard like leather. As the swelling of the testicles subsided rapidly the indurated scrotum became wrinkled. More or less marked ulcerations appeared covered by dry, necrotic epidermis. Also on the indurated vulva and prepuce superficial ulcerations appeared. In one male the penis became swollen and indurated, and there were ulcerations covered by a mucopurulent discharge.

A third quite constant symptom of the general infection was an indurated edema around the base of one or both ears. In some animals it was only slight, in others so pronounced that the ear began to droop. The skin over the swellings became dry and the hair fell out. Also here superficial ulcerations covered by dry crusts appeared in nearly all animals. Indurated swelling of the lips, especially of the upper one, and of the nose was a fairly constant finding. The swelling of the nose appeared generally later than the swelling of lips and genitals. In one animal it was so pronounced that it resulted in a prolongation of the profile. Irregular superficial ulceration and crust formation appeared also here. There was some clear discharge from the nose which soon became mucopurulent, and the margins of the nares were covered by dry crusts under which the skin showed cracks and rhagades. All these lesions of the skin were eminently chronic or

disappeared and reappeared for weeks or months leaving the skin dry and hard and completely devoid of hair. Some lesions showed a tendency to heal in the center, spreading slowly in the periphery. In two animals there was a tumor-like infiltration of the upper lips covered by superficial ulcerations. In only one animal skin lesions were found over the femur, and in one over the back. Here also the lesions started as deep indurations of the cutis with secondary involvement of the epidermis. In repeated instances there occurred a rapid regression of those lesions from which excisions were made; the parts of the lesions immediately surrounding the excision showed marked tendency to heal, whereas the periphery was much less affected. When these swellings on the head and genitals appeared, the pectoral and axillary lymph glands of all animals became palpable. Often they were quite hard and large and they remained so as long as the animals lived.

The general condition of the rabbits remained good during the first 4 weeks after inoculation. When the swellings around the eyes and the soft parts of the genitals were at their height, there was a slight fever in most of the animals. Four succumbed in the 5th week; shortly before death they had marked hyperpyrexia, a profuse watery discharge from the nose, and slight diarrhea. They lost rapidly in weight and died in a comatose condition with convulsions. The remaining six animals lived between 3 and 6 months. Two died as did the four just mentioned, three were killed, and one succumbed during ether anesthesia.

3. Demonstration of the Spiral Organism in the Peripheral Blood, Skin Lesions, and Lymph Nodes.

The organisms which were always found quite easily by dark-field examination in serum aspirated from the primary lesions could not be found by this method either in the peripheral blood or in serum of the secondary lesions of the skin.

Special care was taken to exclude coincident infection with *Treponema cuniculi.* This organism was never found in any of the lesions described. As soon as swelling and infiltration of the eyelids appeared, 0.5 cc. of blood was taken from an ear vein and injected subcutaneously into guinea pigs. At the same time aerobic and anaerobic blood cultures were taken. They remained sterile. After 5 to 7 days the inoculated guinea pigs developed typical primary lesions with numerous spirochetes and all succumbed to the infection, showing the organisms in the blood. The amount of blood necessary for transmitting the organisms to guinea pigs was determined with the blood of two rabbits. The experiment was done at the end of the 3rd week after inoculation. Increasing amounts of blood

were injected into the scrotum or vulva of two sets of guinea pigs. The result of this experiment is given in Table I.

The experiment demonstrates that a measurable amount of blood is necessary for the induction of a primary lesion, and that when little is given the infection tends to be relatively chronic.

TABLE I.

Experiment 1.

Guinea pig No.	Amount of blood injected.	Results.
		Rabbit 1.
	cc.	
1	0.05	No primary lesion; spirochetes in blood after 17 days; chronic course of the infection. Died on the 34th day.
2	0.1	Primary lesion after 8 days; spirochetes in blood after 13 days. Died on the 28th day.
3	0.2	Primary lesion after 6 days; spirochetes in blood after 12 days. Died on the 26th day.
4	0.5	Primary lesion after 5 days; spirochetes in blood after 10 days. Died on the 27th day.
		Rabbit 2.
1	0.05	No primary lesion; no spirochetes in blood but inguinal lymph nodes large on the 12th day.
2	0.1	No primary lesion; spirochetes in blood after 19 days. Very chronic course; lived 41 days.
3	0.2	Small primary lesion after 7 days. Died after 36 days. Spirochetes in blood on the 13th day.
4	0.3	Primary lesion after 6 days; spirochetes in blood on the 11th day. Lived 29 days.
5	0.5	Pronounced primary lesion with deep ulceration after 6 days. Spirochetes in blood on the 11th day. Died on the 26th day.

The time during which the blood of the rabbits is infectious for guinea pigs was determined with the blood of two animals. The results of the experiment showed that the spirochetes slowly disappear from the blood of the animals (Table II).

The experiments illustrated in Tables I and II show clearly that the spiral organism is able to produce a general blood infection in the rabbit. It remained to prove that the skin lesions described were due to a tissue infection with the same organism.

Since the serum taken from these lesions was uniformly negative on microscopical examination, I had recourse to experiments with guinea pigs. Because of the fact that in the first 3 to 5 weeks of general infection only a relatively small amount of blood is necessary to transmit spirochetes from rabbits to guinea pigs,

TABLE II.

Experiment 2.

Guinea pig No.	Length of time after inoculation of rabbit.	Amount of blood injected.	Results.
			Rabbit 3.
	days	*cc.*	
1	13	0.5	Primary lesion after 5 days; spirochetes in blood on the 11th day. Died on the 26th day.
2	20	0.5	Small primary lesion after 6 days; spirochetes in blood on the 12th day. Died on the 28th day.
3	27	0.5	No primary lesion; spirochetes in blood on the 16th day. Died on the 34th day.
4		1.0	Slight edema and induration on the 9th day; general infection on the 15th day. Died on the 33rd day.
5	41	0.5	Remained well.
6		1.0	" "
7		2.0	Remained well but inguinal lymph gland markedly enlarged on the 16th day.
			Rabbit 4.
1	12	0.5	Primary lesion on the 4th day; spirochetes in blood on the 11th day. Died on the 29th day.
2	19	0.5	Primary lesion on the 7th day; spirochetes in blood on the 14th day. Died on the 34th day.
3	26	0.5	No primary lesion; spirochetes in blood on the 15th day. Died on the 39th day.
4		1.0	Very small primary lesion on the 9th day; general infection on the 13th day. Died on the 34th day.
5	33	0.5	No primary lesion; remained well.
6		1.0	" " " " "
7		2.0	No primary lesion; no spirochetes in blood; inguinal lymph nodes hard and large after 15 days.

positive results at this time with tissue excised from the lesions and implanted into guinea pigs are not absolutely convincing. The experiments were therefore repeated with the tissues of animals whose blood no longer transmitted the spirochetes to guinea pigs. Lymph nodes, pieces of testicles, and pieces of skin contain-

ing lesions were removed, thoroughly crushed with sterile forceps and implanted subcutaneously into guinea pigs. Control guinea pigs received a piece of apparently normal skin. The implants were made under aseptic precautions into the lower abdominal wall and the animals were kept separate.

The implantation of crushed lymph glands and pieces of the testicles was always followed after 5 to 9 days by a primary lesion containing numerous spirochetes; and the guinea pigs developed a general infection of which they died. Of ten guinea pigs inoculated with skin lesions five developed a primary lesion containing spirochetes within 7 to 11 days. Four died from general infection within 2 months. The fifth animal remained well but the inguinal lymph nodes were found to be hard and very large 3 weeks after inoculation. No spirochetes were found in the blood, but implantation of the lymph nodes into another guinea pig was followed by a small but typical primary lesion and this animal died from general infection, showing numerous spirochetes in the blood.

Two guinea pigs died from general infection without having developed primary lesions. The course in these two animals was prolonged and they lived 10 and 11 weeks. Two guinea pigs remained well, no spirochetes being found in their blood on numerous careful examinations. The inguinal lymph glands did not become enlarged, and implantation of them into other animals was not followed either by a primary lesion or by general infection. The remaining, or tenth, guinea pig, inoculated with material of a skin lesion developed a very small but distinct primary lesion on the 9th day. Spirochetes were easily found in serum aspirated from it. The inguinal lymph nodes became definitely enlarged and hard but the animal did not develop a general infection and spirochetes could not be found in its blood. 2 months later the inguinal lymph glands were still markedly enlarged but the animal had gained in weight and did not show any clinical signs of illness. The glands were removed, thoroughly crushed, and implanted into two other guinea pigs both of which developed edema and inflammation around the implanted piece after 6 days and subsequently died from general infection with numerous spirochetes in the peripheral blood. Four animals implanted with apparently normal skin taken from the same rabbits used for the experiments just mentioned, remained free of spirochetes.

Series 2.

4. The Transmission of the Spiral Organism from Rabbit to Rabbit by Sexual Intercourse.

Six rabbits were used in this experiment. Two non-pregnant females and one male of Series 1 were mated with two males and one non-pregnant female. The pairs were kept separated. The healthy animals (Series 2) had been examined previously for lesions due to *Treponema cuniculi*. No such lesions were found. The experiment was successful in one instance.

One male showed 15 days after mating an edematous swelling of the prepuce and sheath which in a few days became much inflamed. The lesion resembled very much the primary lesion described above, and there developed deep ulceration around the orifice of the sheath. The penis became very swollen also and could not be put back into the sheath. In serum aspirated from the edematous swelling the rapidly moving spirochetes could easily be demonstrated. The animal developed a general infection, with pronounced swelling of eyelids, lips, testicles, and bases of the ears, and the organisms were demonstrated in its blood by experiments with guinea pigs. The much enlarged pectoral lymph nodes and pieces of tissue removed from ulcerated lesions on the neck and base of the right ear when implanted into guinea pigs 8 months after the observation of the primary lesion, transmitted the spirochetes, whereas blood injected at this time in amounts up to 5 cc. into guinea pigs proved innocuous. The rabbit died 10 months after inoculation. The two other rabbits exposed remained well, and repeated attempts to transmit the spirochetes to guinea pigs by means of their blood all failed. None of the females became pregnant.

5. Demonstration of Immune Bodies in the Blood of the Rabbits.

The fact that pieces of the cutaneous lesions and enlarged lymph nodes of the male infected by sexual intercourse contained the spirochetes 8 months after infection, whereas they could not be demonstrated in the blood even by a transmission experiment, suggested the presence of immune bodies in this fluid. The serum was tested against spirochetes which were obtained in large numbers from a well developed primary lesion of a guinea pig.

There were ten to twelve spirochetes in one optical field in the tissue juice squeezed out after puncture with a Hagedorn needle. Blood serum of a healthy rabbit served as a control. A loopful of the supposedly immune serum was mixed with a loopful of the juice obtained from the primary lesion and examined

immediately with the dark field. The spirochetes were clearly seen in progressive movements for a few seconds when most of them suddenly became motionless, whereas a few were still seen turning rapidly around the transverse axis to become motionless a few moments later. In less than 1 minute the spirals of most of the organisms had disappeared; the body swelled, elongated, and filled with refractile dots; and some organisms broke up completely. In serum diluted 1:2 most of the spirochetes became motionless within 5 minutes, a few still showed undulating movements, but their structure was not affected. In serum diluted 1:4 no marked influence was noticed. In the control test with normal undiluted rabbit serum the organisms moved freely across the field for 20 minutes, when their movements became slower.

TABLE III.

Experiment 3.

Intraperitoneal Injection of Immune Serum-Spirochete Mixture Immediately after Mixing.

Guinea pig No.	Concentration of immune serum.	Amount injected.	Results.
		cc.	
1	0.5 cc. blood + 0.5 cc. immune serum.	0.5	Spirochetes in blood after 17 days. Died on the 36th day.
2	0.5 cc. blood + 0.25 cc. immune serum + 0.25 cc. saline.	0.5	Spirochetes in blood after 15 days. Died on the 32nd day.
3	0.5 cc. blood + 0.2 cc. immune serum + 0.3 cc. saline.	0.5	Spirochetes in blood after 12 days; small primary lesion in abdominal wall. Died on the 29th day.
4	0.5 cc. blood + 0.1 cc. immune serum + 0.4 cc. saline.	0.5	Spirochetes in blood after 11 days. Died on the 29th day.
5	0.5 cc. blood + 0.05 cc. immune serum + 0.45 cc. saline.	0.5	Spirochetes in blood after 12 days; small primary lesion on abdominal wall. Died on the 26th day.
6 Control.	0.05 cc. blood + 0.95 cc. saline.	0.5	Spirochetes in blood on the 11th day. Died on the 26th day.

The protective power of serum shown in this way to injure the spirochete was tested in two experiments. The spirochete-containing blood was taken from the heart of a guinea pig. On examination it showed numerous organisms. It was mixed with decreasing amounts of immune serum containing 0.2 per cent of sodium citrate, the volume being completed with sterile saline. One part of the mixture was incubated for 15 minutes at 37° and then injected subcutaneously into guinea pigs, whereas the other part was injected immediately

after mixing into the peritoneal cavity of another series of guinea pigs. Control animals received one-tenth the amount of infective blood without the addition of immune serum.

Tables III and IV give the results of these experiments.

Whereas all animals of Experiment 3 showed clinical signs of illness after 16 days, with large, hard inguinal glands, the animals of Experiment 4 did not show any clinical signs of illness at this time but the regional lymph glands of Animals 3 and 4 were found to be slightly

TABLE IV.

Experiment 4.

Subcutaneous Injection of Immune Serum-Spirochete Mixture after 15 Minutes Incubation at 37°.

Guinea pig No.	Concentration of immune serum.	Amount injected.	Results.
		cc.	
1	0.5 cc. blood + 0.5 cc. immune serum.	0.5	No primary lesion; no spirochetes in blood; remained well but inguinal lymph nodes became enlarged.
2	0.5 cc. blood + 0.25 cc. immune serum + 0.25 cc. saline.	0.5	No primary lesion. Died of general infection.
3	0.5 cc. blood + 0.2 cc. immune serum + 0.3 cc. saline.	0.5	" "
4	0.5 cc. blood + 0.1 cc. immune serum + 0.4 cc. saline.	0.5	Primary lesion after 12 days. Died of general infection.
5 Control 1	0.05 cc. blood + 0.95 cc. normal rabbit serum.	0.5	Primary lesion on the 5th day; spirochetes in blood on the 11th day.
6 Control 2	0.05 cc. blood + 0.95 cc. saline.	0.5	Primary lesion on the 4th day; spirochetes in blood on the 11th day.

enlarged. In Animal 2 they became palpable on the 18th day and in Animal 1 on the 21st day. All animals except No. 1 developed a general infection with well marked skin lesions. No. 1 never showed any spirochetes in the blood and was still well 2 months after injection. The lymph glands of one side were removed and implanted into a healthy guinea pig which developed a primary lesion and a general infection with numerous spirochetes. The experiment shows that the serum was able to prevent the development of a primary lesion in

guinea pigs in dilutions of 1:2 to 1:4 and 1:5, but that the protective principle it contained was not strong enough to protect the animals from a general infection, although Animal 1 developed only a latent infection. It shows also that the time during which the immune serum was left in contact with the spirochetes was very important. To elaborate the point a further test was made. 1.5 cc. of immune serum was used and 1.5 cc. of fresh normal rabbit serum. To both samples a drop of tissue juice taken from a well developed primary lesion of a

TABLE V.

Experiment 5.

Guinea pig No.	Mixtures.	Amount injected.	Results.
No. 1		*cc.*	
Right side of abdominal wall.	1.5 cc. immune serum + 0.05 cc. spirochetes.	0.5	No primary lesion developed at the site of inoculation.
Left side of abdominal wall.	1.5 cc. normal rabbit serum + 0.05 cc. spirochetes.	0.5	Primary lesion after 5 days at the site of inoculation. Died of general infection.
2	1.5 cc. immune serum + 0.05 cc. spirochetes.	0.5	No primary lesion; remained well.
3	1.5 cc. immune serum + 0.05 cc. spirochetes.	0.5	" "
4	1.5 cc. normal rabbit serum + 0.05 cc. spirochetes.	0.5	Primary lesion after 4 days. Died of general infection.
5	1.5 cc. normal rabbit serum + 0.05 cc. spirochetes.	0.5	Primary lesion after 6 days. Died of general infection.

guinea pig, and containing large numbers of spirochetes, was added and the mixture incubated at 37°C. for 30 minutes. To exclude the factor of individual differences as affecting the development of a primary lesion one animal was injected in the right part of the shaved abdominal wall with 0.5 cc. of immune serum-spirochete mixture, and in the left part with 0.5 cc. of normal serum-spirochete mixture. Two other animals received 0.5 cc. of immune serum-spirochete mixture, and two animals 0.5 cc. of normal serum-spirochete mixture.

Table V gives the results.

6. *Superinfection.*

The circumstances that the blood serum of an animal proved spirocheticidal while it still harbored virulent spirochetes in the skin and in the lymph nodes would *a priori* seem not at all incompatible with a local superinfection. But it proved impossible to superimpose a lesion of the skin in this animal despite the injection of material very rich in organisms, and repeated trials in different parts of the body. Eventually the cornea was chosen for test. An inoculation into the skin is not feasible without injuring blood vessels, with result in a subsequent effusion of blood into the tissues. In the cornea, however, this does not happen.

The rabbit with the blood of which Experiments 3, 4 and 5 had been carried out was the animal employed. It was inoculated in the upper segment of both corneæ with blood taken aseptically from a guinea pig. The inoculations were made with a very fine glass tube, to cause the smallest possible lesions. Two healthy control rabbits were injected in the same part of one cornea with the same amount of infective blood. Prior to the injection the conjunctival sac was in each case carefully rinsed out with sterile saline solution.

Keratitis developed in all four injected eyes. This was evidenced in the beginning by a slight milky cloudiness extending from the upper margin of the cornea to the place of inoculation. There was intense injection of the vessels over the musculus rectus superior accompanied by swelling and reddening of the conjunctivæ of the upper lids. There was, however, a difference in the experimental animal and in the two controls. In the former, keratitis was noticeable in the right cornea after 48 hours, in the left after 72 hours; whereas in the controls it appeared on the 4th and 5th days respectively. The keratitis in the left eye of the blood-immune animal disappeared after 5 days, and in the right after 7 days, whereas in the controls it lasted for 10 and 12 days and was followed by general infection with the clinical manifestations already described.

7. *Distribution of the Organisms in the Body of the Rabbit.*

As already mentioned the spirochetes could always be found by direct examination of material aspirated from the primary lesions; but it was generally necessary to search carefully for them, as they were never present in as large numbers as in the primary lesions of guinea pigs. In the peripheral blood they were so few that I never succeeded in finding them with the dark field, but their presence could always be demonstrated by the injection of blood into guinea pigs.

They disappeared gradually from the blood stream during the course of the infection but could be recovered from the tissues many months after the infection had taken place.

In material taken from the secondary lesions the results of dark-field examination were uniformly negative, and in lesions treated by Levaditi's method I found the organisms only after tiresome search, during which numerous sections were examined. I never succeeded in finding the organisms in the granulation tissue or the infiltrated foci of the skin lesions, but only in the connective tissue immediately surrounding the infiltrated parts. I experienced the same difficulty in finding them in the enlarged lymph nodes. The scarcity of the organisms in the tissues of the rabbits may best be illustrated by the fact that in the lymph glands of one animal, small pieces of which transmitted the organisms to guinea pigs, they could not be found in Levaditi preparations. In one animal I found a few organisms in the upper segment of the cornea near the sclera. I found a very few of the spirochetes in the organs (except the brain, spinal cord, and lung) of one animal which had died in the 5th week after infection. None could be found in the viscera of two other animals which were killed after the disease had endured more than 2 months.

Emulsions of the internal organs of two rabbits were injected into guinea pigs. One of the rabbits had died from ether anesthesia 9 weeks after infection. The blood of this animal injected in an amount three times larger than the amount of emulsion of the organs injected, did not transmit the spirochetes to guinea pigs, whereas all guinea pigs injected intraperitoneally with the emulsion made from spleen, liver, kidneys, and adrenals of the same animal developed a very chronic infection. Another series of guinea pigs was injected with emulsion of the viscera of a rabbit which had died 10 months after inoculation. As the undiluted serum of this animal had protected guinea pigs against the infection with the spirochetes, the viscera were cut into very small pieces, thoroughly washed with plenty of sterile saline, then emulsified and added to five volumes of saline before injection. None of the five guinea pigs injected developed clinical signs of illness during 2 months of observation, but the spirochetes were demonstrated in the slightly enlarged lymph nodes of one animal by injection of them into guinea pigs.

8. Latent Infection in Guinea Pigs.

Several guinea pigs did not develop clinically manifest symptoms of the infection, but the organisms were recovered from them after

many weeks by implantation of the inguinal lymph nodes into other guinea pigs. Evidently here was a latent infection, by which we understand a condition wherein the infective organisms and the infected host live in equilibrium. Several of the guinea pigs which harbored spirochetes in the lymph nodes, without having developed any clinical signs of illness, were kept under observation for a long time. Frequent examinations of their blood gave only negative results. Finally they were kept together for lack of room. Among these animals was a female which became pregnant. The pregnancy progressed normally, nearly reaching its end, when the animal suddenly developed edema and induration of the eyelids on both sides followed by keratitis on one side. Examination of the blood then showed numerous spirochetes and the animal had a miscarriage. Unfortunately the fetuses and placenta were almost entirely eaten. The mother died shortly afterwards with typical signs of a general infection. The remaining animals were killed 4 months after the spirochetes had been found in the inguinal lymph nodes. Emulsions of the organs and lymph nodes no longer transmitted spirochetes to other guinea pigs.

9. Comparison of the Rat Strain with a Human Strain.

The admittance of a case of rat-bite fever to the American Hospital, Mexico City, gave me the opportunity to compare the organism isolated from this patient with the organism isolated from a wild rat and the subject of the present paper. The morphology of both strains was strikingly similar and the subcutaneous injection into guinea pigs of the human strain was followed after 3 to 5 days by a local edema and subsequent hard induration. The lymph nodes were always found to be enlarged and after a few passages through guinea pigs the general infection was always accompanied by the eye and skin lesions observed in guinea pigs inoculated with the rat strain. The human strain also proved pathogenic for rabbits, inducing in these animals the same clinical picture described in this paper for the rat strain. A mixture of undiluted rat strain immune serum with tissue juice from a primary lesion of a guinea pig produced by the human strain, failed to transmit the disease to guinea pigs; and dilutions of the rat strain serum in many instances prevented the development of a

primary lesion though not the general infection. Experiments could not be carried out with the serum of the patient since he was treated with salvarsan.

10. Pathological Findings in Rabbits.

All animals that died or were killed were carefully examined as routine.

(*a*) *Macroscopic Changes.*—The animals which were killed did not reveal any gross changes in the viscera. There was some hyperemia and cloudy swelling of the parenchymatous organs. Contrary to the findings in guinea pigs the spleen was never enlarged. Cultures from the heart and from the spleen proved sterile. The testicles were always quite hard and atrophic. The animals which died of intercurrent disease showed a slightly enlarged spleen, and small bronchopneumonic foci in the hyperemic lungs; one had a purulent pericarditis and two a purulent pleurisy. Bacteriological examination of these animals revealed three times a bacillus of the Pasteurella group, twice a colon bacillus, and once an organism of the Friedländer group. Examination of the animal which died suddenly after 10 months duration of the infection revealed a pronounced fibrosis of the myocardium especially of the left ventricle. Cultures taken from this animal remained sterile.

(*b*) *Microscopic Changes.*—The microscopic examination of the viscera only was made on tissue from rabbits which had been killed. It did not reveal any gross changes except in the testicles. There was some cloudy swelling and hyperemia of the liver and the cortex of the kidneys. In the interstitial tissue of the lungs, liver, kidneys, and adrenals small infiltrations with lymphocytes, plasma cells, large mononuclear cells, together with a proliferation of fibroblasts, were often found. Similar cell collections were found around small arteries of the myocardium in two animals. The pulp of the spleen showed a slight increase in polynuclear cells. Occasional nucleated red cells, erythrophagy by endothelial cells, and sometimes hemosiderin deposits were found.

Brain and Spinal Cord.—Normal.

Testicles.—The soft intertubular stroma was found to be replaced by cell-rich granulation tissue formed of fibroblasts, lymphocytes, large mononuclear cells, and great numbers of small blood vessels. Numerous collagen fibrils indicated the tendency to fibrosis. The tunica albuginea was much thickened and infiltrated by lymphocytes and plasma cells collected chiefly around small vessels. The epithelial lining of many tubules had disappeared nearly completely and the lumina were filled by a mucous-like exudate and desquamated epithelial cells showing more or less complete karyorrhexis and karyolysis. Many tubules were found to be replaced completely by granulation tissue.

Cornea.—There was present a marginal infiltration in the upper segment by lymphocytes, plasma cells, and large mononuclear cells.

Striated Skeletal Muscles.—Slight atrophy; seldom a hyaline, never a waxy degeneration.

Lymph Nodes.—Hyperemia, hyperplasia, and desquamation of endothelial cells, especially in the marginal sinus.

The Primary Lesion.—In the early lesion there was pronounced edema and hyperemia accompanied by extravasation of polynuclear cells reaching from the epidermis far down into the cutis and subcutis. On the 3rd day after the appearance of the lesion there was still marked edema and a very dense infiltration with polynuclear leucocytes, lymphocytes, plasma cells, and large mononuclear cells. Extravasations of red cells were often surrounded by eosinophilic leucocytes. Later the connective tissue cells began to proliferate freely, mixing diffusely with the exudate cells. Many small blood vessels showed swelling and proliferation of the intima, and the adventitia and media were often densely infiltrated with lymphocytes, plasma cells, and large mononuclear cells. Finally the lesion presented a granulation tissue with numerous small blood vessels. Where superficial ulceration had taken place there was a dense wall of polynuclear leucocytes surrounding the ulcer.

The Secondary Lesion.—The character of the secondary lesion was essentially the same as that of the primary lesion. The difference lay principally in the quicker replacement of the exudate by the granulation tissue. There was pronounced infiltration and proliferation of the small blood vessels with extravasation of blood, or only plasma. Occasionally small, distinct nodules formed by epithelioid cells were found in the secondary lesions. The presence of giant cells of the Langhans type sometimes gave them a close resemblance to a miliary gumma. Also these secondary lesions involved nearly always the whole thickness of the skin, infiltrating the subcutis and the muscular layer of the platysma and facial musculature. Some lesions showed dense perivascular collections of lymphocytes and plasma cells. The lesions are easily distinguishable from those produced by *Treponema cuniculi*, which are very superficial, consisting of collections of lymphocytes and plasma cells, chiefly in the interpapillary spaces, without conspicuous lesions of the blood vessels.

DISCUSSION.

The experiments demonstrate that the rabbit is susceptible to infection with the spiral organism reported upon in an earlier paper. Subcutaneous inoculation of blood containing the spirochetes is followed after 3 to 5 days by local edema, induration, and inflammation occasionally leading to ulceration,—the primary lesion. After a further period of incubation (about 8 days from the appearance of the primary lesion) signs of a general distribution of the organisms appear. There is a more or less pronounced puffiness of the face followed soon by edema of the eyelids, lips, nose, bases of the ears, and genitals, and

especially of the testicles. These edematous parts soon become infiltrated and hard; and superficial ulcerations of the skin are often met with. At the same time the pectoral and axillary lymph nodes become hard and easily palpable,—the secondary lesions. Whereas the spirochetes can always be found quite easily in tissue juice from the primary lesions by dark-field examination, their presence in the blood and in the secondary lesions can generally be demonstrated only by injection of the material into guinea pigs. The organisms disappear from the blood completely during the course of the disease. The course of the infection is in this respect different from that observed in guinea pigs, in which the spirochetes can always easily be demonstrated in the peripheral blood throughout the course of the disease. This difference is due to the formation of spirocheticidal antibodies in the blood of the rabbit. In spite of the disappearance of the spirochetes from the blood of rabbits, doubtless on account of these antibodies, the organisms can still be isolated from the skin lesions, the lymph nodes, and the viscera. It seems, therefore, that the antibodies do not pass intact blood vessels in sufficient quantity to kill the organisms in the tissues. The observation of a rapid regression of skin lesions from which excisions have been made is easily explained by the effusion of immune blood into the tissue through the injured blood vessels and the same explanation will suffice for the not infrequent occurrence of annular lesions in the animal with a blood immunity. Histological study of such lesions reveals pronounced injuries of the wall of the blood vessels which lead to effusion of blood or plasma into the tissues. At the center, which is the oldest part of the lesion, the vessels are injured first with result in effusion of antibodies, destruction of the spirochetes, and subsequent local regression of the lesion, whereas at the periphery, to which the effused blood does not reach, the lesion may still spread. It is not surprising that organisms are so scarce in the infiltrated skin lesions, and that they can generally be found only in the immediate surroundings of the infiltrated parts. That it is possible to induce a keratitis in a blood-immune animal is easily explained by the fact that the cornea has no blood vessels.

The development of a primary lesion seems to depend chiefly on the amount of spirochetes introduced. Guinea pigs injected with

relatively large amounts of blood from rabbits at the height of the disease invariably develop a primary lesion and a subsequent easily demonstrable general infection, whereas small amounts of the same blood frequently fail to produce a primary lesion, although giving rise usually to a general infection. A latent infection is observed only in guinea pigs which receive very small numbers of spirochetes. The injection of very small amounts of infective blood or tissues has the same relatively slight effect as that of material rich in spirochetes but treated with immune serum that has not sufficient strength to kill all the organisms present. This fact must be kept in mind in looking for the spirochetes in an advanced case of rat-bite fever in which the blood may contain very few spirochetes. The injected animals may develop only a latent infection from which the spirochetes can probably be isolated only by the injection of the inguinal lymph nodes into other animals.

In my first paper on this spirochete I expressed the opinion that it is identical with the organism isolated first by the Japanese from cases of human rat-bite fever. This assumption I based on the morphological similarity of the organisms and the results of experiments on white rats and guinea pigs. The fact that I was able to infect rabbits with the organisms might be thought to open some doubts in this respect, but the experiment which has demonstrated that the rat strain immune serum is able to protect guinea pigs against infection with the human strain, furnishes evidence to justify the conclusion that the human strain and the rat strain isolated in Mexico City are identical. It is more than probable that this spirochete has a world-wide distribution, as I isolated it from a *Mus decumanus* which is a well known traveller. Investigators who work with rats should always be aware of the possibility of encountering this organism. The clinical picture in guinea pigs and rabbits infected with it is so strikingly similar to the picture given by Brown and Pearce of rabbits and guinea pigs infected with certain strains of *Trypanosoma*, that their photographs could easily be replaced by some of the photographs of my animals. The observation of a very similar spirochete in a white mouse experimentally infected with *Trypanosoma dimorphon* by Breinl and Kinghorn deserves mention, because it is very well possible that the organism I am dealing with may occasionally be found in white rats.

In my first paper I classified the organism in the genus *Treponema* on account of its morphology. The experiments with guinea pigs, and especially those with rabbits reveal a close similarity between the course of the infection induced by this organism and experimental syphilis. There occurs in both cases a primary lesion after a period of incubation followed by a general distribution of the organisms through the blood stream and the appearance of secondary lesions after a second period of incubation. The observation of a latent, *i.e.* clinically not manifest but apparently chronic infection, further enhances the similarity. In this connection one may recall the close morphological resemblance of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* and the similar clinical picture they produce in the infected host. The spiral organism of rat-bite fever should be classified definitely under the genus *Treponema*. The etiology of rat-bite fever has been definitely established by the work of the Japanese investigators and their findings have lately been confirmed repeatedly outside of Japan. There have been recorded, however, many cases in which other organisms than that described in the present paper have been deemed responsible for the disease. *Aspergillus*, streptothrix, diplococci, and bacteria were found in these cases. Several of my rabbits infected with the treponema died from bacterial invasion of the blood and it is possible that the infection with the spiral organism invites invasion by other organisms.

CONCLUSIONS.

1. The spiral organism isolated from a wild rat has proved identical with that from a human case of rat-bite fever in Mexico City.

2. Rabbits can easily be infected with these organisms.

3. The subcutaneous inoculation of them is followed after 3 to 5 days by a local edema, induration, and inflammation: primary lesion.

4. About 8 days after the appearance of the primary lesion, edematous swellings and inflammation appear on the head and genitals: secondary lesions.

5. The organism can be transmitted from rabbit to rabbit by coitus.

6. Protective antibodies are to be found in the blood of infected rabbits at a time when the local lesions are still active. The peculiar

character of certain of the lesions is probably due to the escape of the antibodies from the blood stream.

7. The organism belongs to the genus *Treponema*.

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EXPLANATION OF PLATES.

PLATE 24.

FIG. 1. Primary lesion on prepuce.

FIG. 2. Swelling and infiltration of the eyelids and back of the nose.

FIG. 3. Swelling and infiltration of eyelids; swelling and ulceration on base of the ear.

PLATE 25.

FIG. 4. Pronounced swelling of vulva and anus. Secondary lesion.

FIG. 5. Swelling of the testicles. Superficial ulceration and necrotization of the scrotum.

FIG. 6. Swelling of penis, prepuce, and anus (secondary lesion). The swelling of the testicles had already subsided, showing a wrinkled ulcerated scrotum.

(Mooser: Spiral organisms in wild rat. II.)

(Mooser: Spiral organisms in wild rat. II.)

BACTERIOPHAGE TESTS ON THE MECONIUM OF ABORTED FETUSES.

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Since d'Hérelle's[1] advocacy of the living nature of the bacteriophage there has been a divergence of opinion as to its real nature and origin, although its widespread prevalence in the intestinal tract of adult man and animals meets with general acceptance. The stimulus for the present work lay in the hopes that it might add further information to the theory that bacteriophagous lysis can be initiated by tissue cells.

Bordet and Ciuca[2] were among the first to question the living nature of the bacteriophage, for by injecting *Bacillus coli* intraperitoneally into guinea pigs they demonstrated that the resulting exudate could bring about transmissible lysis of the injected strain. The source of the lytic substance was attributed to the leucocytes.

Wollstein[3] and Kuttner[4] have repeated these tests with some success, although it appears that this method is not without its difficulties. Human peritoneal exudates examined by Wollstein contained a *coli* phage in a case of peritonitis and a dysentery phage in a case of fatal dysentery. Kuttner reports the extraction of phage from the intestinal mucosa and liver cells of normal guinea pigs, as well as demonstrating its presence in normal rabbit serum. Callow[5] isolated phage from the lesions in sixteen cases of staphylococcus infection, and McKinley[6] from seventeen different sources in normal and pathological tissues of man and animals.

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[1] d'Hérelle, F., authorized translation by Smith, G. H., The bacteriophage. Its rôle in immunity, Baltimore, 1922.

[2] Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293.

[3] Wollstein, M., *J. Exp. Med.*, 1921, xxxiv, 467.

[4] Kuttner, A. G., *J. Bact.*, 1923, viii, 49.

[5] Callow, B. R., *J. Infect. Dis.*, 1922, xxx, 643.

[6] McKinley, E. B., *J. Lab. and Clin. Med.*, 1923–24, ix, 185.

The impression to be gathered from the interpretations of these investigators is that the tissue cells may play a rôle in the production of bacteriophage. Objections to the leucocyte theory of Bordet and Ciuca were early made by d'Hérelle on the basis of his previous experiments indicating that the bacteriophage could penetrate the intestinal mucosa and migrate by way of the blood stream throughout the body. Its presence in peritoneal exudates he holds is in accordance with this phenomenon. All experiments having to do with tissue cells as a source of bacteriophage are subject to a like interpretation in the presence of an intestinal tract with complex bacterial and associated bacteriophagous flora.

The study of cattle abortion as carried on in this laboratory appeared to offer material admirably adapted for the elimination of the above objection, since the flora of the rectal contents of aborted fetuses was usually reduced to its simplest terms; namely, *Bacillus abortus*.

Six fetuses ranging in age from 6 months to approximately full term were tested. Any which had breathed and from which an organism other than *B. abortus* was recovered from the rectal contents were discarded. The organism was not isolated from one of the included cases, although as it was recovered from the uterine discharges of the dam it was undoubtedly present. 16 to 20 gm. of meconium were removed aseptically to 30 to 100 cc. of sterile fermented bouillon, shaken well, centrifugalized, passed through a Berkefeld filter, and then placed in the ice box until used. The filtrates diluted 1:10 in slightly alkaline bouillon were tested against twelve strains of *B. abortus* isolated in this laboratory, of which three were derived from the above fetuses. One of the latter failed to grow in bouillon, as did one of the other strains. Inoculum into the diluted phage was 0.5 cc. of a 3 day old bouillon culture. After a 24 hour incubation at 37°C., 0.5 cc. quantities were inoculated onto the surface of slightly alkaline agar slants. Control tubes without the addition of filtrate were used for each culture. All tubes were examined up to a week for evidence of lysis. In no instance, however, was there any trace of lysis to be observed.

In view of these frankly negative results, it was decided to test the filtrates against bacteria other than *Bacillus abortus*. For this purpose a number of different strains of colon bacilli isolated in this laboratory from various calf conditions were used,—34 in all, although no filtrate was subjected to the whole list. A lysable strain of human origin was also included.

In these tests the filtrates were diluted 1:10 with alkaline bouillon and inoculated with one loopful of an emulsion made from an 18 hour culture grown on slanted agar. Immediately following inoculation, 0.5 cc. quantities were spread over the surface of agar slants. Culture controls without filtrate were included. Incubation was at 37°C. At no time up to 6 days was there any evidence of lysis, although the *B. coli* human strain in the presence of its homologous bacteriophage was completely lysed. Sterility tests on the filtrates yielded no growths.

DISCUSSION.

In so far as these tests on meconium show, there is no indication that the fetuses produced a bacteriophage in the presence of a specific infection, or that one was naturally present for numerous colon strains of bovine origin. That fetal tissues are capable in other respects of reacting against *Bacillus abortus* has been indicated recently by Theobald Smith[7] in studies on fetal pneumonias.

In association with other work the writer has been able to demonstrate in the rectal contents of young calves a bacteriophage active for bovine strains of colon bacilli as well as for the human type used above.[8]

SUMMARY.

The theory that tissue cells are the source of bacteriophage was objected to by d'Hérelle on the grounds that bacteriophage in the intestinal contents could penetrate the intestinal mucosa and migrate throughout the body and that any demonstration of its presence within the body fluids was in accordance with this phenomenon. The present work sought to overcome this objection by using tissues which had been exposed to but a single organism,—*Bacillus abortus*. Filtrates of meconium from six aborted bovine fetuses were tested against several strains of *Bacillus abortus*, but no evidence of a lytic principle could be demonstrated. Neither could it be shown that they contained a substance which would initiate lysis when tested against numerous strains of bovine colon bacilli.

[7] Smith, T., *J. Exp. Med.*, 1925, xli, 639.

[8] Since this work was completed, the writer's attention has been called to a paper by Surányi, L., and Kramár, E., *Monatschr. Kinderheilk.*, 1924, xxviii, 330. In a study of ten new-born infants no bacteriophage active for organisms of the colon-dysentery group could be demonstrated in the meconium, and, furthermore, such a bacteriophage was not detected until after the 14th day of life.

STUDIES ON THE RELATIONSHIP OF THE CHEMICAL CONSTITUENTS OF BLOOD AND CEREBROSPINAL FLUID.

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(Received for publication, June 22, 1925.)

The first part of this report deals with the results of analyses of blood and cerebrospinal fluid from patients in the Henry Phipps Psychiatric Clinic of the Johns Hopkins Hospital. The analyses were made in the Laboratory of Internal Medicine of that clinic, and our thanks are due to the director of that laboratory, Dr. R. S. Lyman, for help and facilities extended to us. As the difficulties of the work with patients became clear, this aspect of the study was brought to an end, and in its place the experimental work on dogs was undertaken in the Department of Physiology of the Johns Hopkins Medical School. We are under obligations to Dr. E. K. Marshall, Jr., and to Dr. A. Grollman for many valuable criticisms and suggestions in this phase of the investigation.

I. Human Material.

In the work on patients a rigid routine was followed. After a 12 hour fast a venepuncture was done in the morning, and immediately after it a lumbar puncture. The blood, for various reasons, was not taken under oil; but to minimize the resultant error the samples of blood were handled identically in all cases. The syringe was emptied into a bottle containing oxalate, where it was gently stirred and corked while the lumbar puncture was being made. Within 15 minutes the blood had been centrifuged. The plasma was then pipetted off at once and the proteins precipitated.

Samples of approximately 15 cc. of the cerebrospinal fluid were always taken. The fluids were counted for cells, tested for globulin, and a Wassermann and colloidal gold test made. They were examined chemically for reducing bodies (Folin and Wu (2)), for chlorides (Whitehorn (7)), and in some cases for the nonprotein nitrogenous constituents (Folin (3)). The same chemical examinations were made upon the samples of venous blood.

TABLE I.

Case No.	Date.	Diagnosis.	Spinal fluid findings.				Chemistry.								
			Cells.	Gold curve.	Globulin.	Spinal fluid Wassermann reaction.	NaCl			Sugar.			N-P N.		
							Blood.	Spinal fluid.	Ratio.	Blood.	Spinal fluid.	Ratio.	Blood.	Spinal fluid.	Ratio.
2605	Feb. 28	Paresis.	5	Paretic.	2+	1+	664	769	1.16	107	59	0.55			
3711	Apr. 7	"	7	"	3+	1+	662	790	1.19	110	73	0.67	32	31	0.93
3781	Jan. 18	"	R.B.C.	"		1+				106	63	0.60			
3789	" 21	Epilepsy.	Negative throughout.							101	73	0.72			
3801	" 22	"	"	"						105	61	0.58			
3805	" 22	Paranoid depression.	"	"						102	65	0.64			
3815	Feb. 15	Multiple sclerosis.	10	Paretic.	1+	Neg.				100	79	0.79			
3821	" 7	Cerebral arteriosclerosis.	Negative throughout.							110	65	0.60			
3827	Jan. 28	Paresis.	13	Luetic.	3+	"				90	53	0.59			
	Feb. 29		4	Lower.	1+	"	645	743	1.15	91	64	0.71			
3829	Mar. 23	Cerebral arteriosclerosis (aphasia).	Negative throughout.				729	771	1.06	123	66	0.53			

3836	Apr. 2	Cerebral arteriosclerosis (delirium).	Negative throughout.				812	941	1.15	165	89	0.53	182	167	0.92
		(Intravenous injection of 300 cc. of 5 per cent glucose given 2 hrs. before the lumbar puncture.)													
3840	Feb. 9	Postencephalitic. Parkinson.	Slightly bloody.			Neg.	652	730	1.12	106	71	0.67			
3842	Apr. 2	Constitutional psychopath.	Negative throughout.				627	738	1.17	91	61	0.67	33*	23	0.69
3849	" 7	Alcoholic delirium (acute).	"	"			660	747	1.13	83	60	0.72	23	15	0.65
3853	Feb. 9	Manic excitement.	"	"			767	730	0.95	102	70	0.68			
3862	Jan. 31	Recurrent depression.	"	"						104	77	0.73			
3869	Apr. 10	Cerebral hemorrhage (2 wks. before).	Bloody. W.B.C.+++			"	530	650	1.22	113	43	0.38	25	30	1.2
	" 12		Less bloody.				514	652	1.27	117	38	0.33	34	39	1.15
	" 29		R.B.C. few. W.B.C. "				587	697	1.18	104	55	0.53	25	17	0.69
3873	May 12	Paresis.	9	Paretic.	3+	4+	635	730	1.15	111	71	0.64	40	31	0.78
3874	Jan. 18	"	11	"	4+	4+				101	55	0.54			
	Feb. 29		5	"	2+	4+	612	771	1.26	95	54	0.57			
	Apr. 29		9	"	3+	?	584	703	1.24	95	56	0.58	32	21	0.65
	May 12		12	"	1+	4+	617	736	1.19	103	69	0.67	28	19	0.67
3885	Mar. 28	Manic; diabetic.	Negative throughout.				600	747	1.26	303	198	0.65	33*	25	0.75
3890	" 28	Cerebral arteriosclerosis.	"	"						133	84	0.63			

* Whole blood, in mg. per 100 cc. All the rest of the figures are for plasma.

TABLE I.—*Concluded.*

Case No.	Date.	Diagnosis.	Spinal fluid findings.				Chemistry.								
							NaCl			Sugar.			N-P N.		
			Cells.	Gold curve.	Globulin.	Spinal fluid Wassermann reaction.	Blood.	Spinal fluid.	Ratio.	Blood.	Spinal fluid.	Ratio.	Blood.	Spinal fluid.	Ratio.
3897	Apr. 10	Tabes, arrested; drug addiction.	Negative throughout.				649	753	1.16	94	61	0.64	40*	30	0.75
3898	" 7	Depression.	"	"			619	722	1.16	91	74	0.81		17	
3916	Jan. 12	Delirium in a schizophrenic.	"	"			107			107	82	0.77			
3917	Mar. 2	Cerebral arteriosclerosis (depression and delirium).	"	"			638	730	1.14	113	73	0.64			
3921	Apr. 10	Paresis.	25	Paretic.	4+	4+	632	726	1.14	97	55	0.56	37*	18	0.51
	June 10		5	"	4+	4+	560	727	1.30	87	69	0.79	26	17	0.65
	July 3		5	"	4+	4+	602	753	1.25	106	63	0.60	18	13	0.69
	" 8		2	"	4+	4+	627	740	1.18	104	65	0.63	22	15	0.68
3985	Feb. 28	Agitated depression.	Negative throughout.				674	764	1.13	90	66	0.74			
51021†	Mar. 26	Uremia.	"	"			568	740	1.30	116	64	0.55	199*	170	0.85
51164†	" 15	Cerebral hemorrhage (4 wks. before).	Still slightly bloody.			Neg.	612	717	1.18	92	49	0.53	22	17	0.79
51168†	May 15	Epilepsy (after 2 days starvation).	Negative throughout.				585	717	1.23	74	60	0.81	30	18	0.59

† Johns Hopkins Hospital Medical Service case numbers. All others are Henry Phipps Psychiatric Clinic case numbers.
All absolute values are in mg. per 100 cc. All ratios are of spinal fluid over blood.

1. The Sugar in Venous Blood and Spinal Fluid.

Table I presents the essential data of all of the patients examined. The ratio of spinal fluid sugar over blood sugar is seen to vary from 0.33 to 0.81. The only ratios, however, which fall below 0.53 occur after cerebral hemorrhage with bloody fluid. For the rest, the range which we encountered was from 0.53 to 0.81, and there is no difference between the fluids with normal and those with pathological findings. (In our series we have no acute or tuberculous meningitis, and no acute encephalitis.)

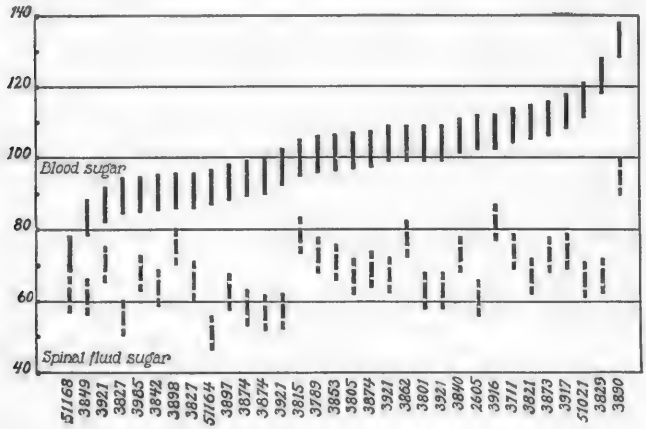

FIG. 1. The blood and spinal fluid sugars from a series of patients, arranged in the order of increasing blood sugars. The vertical bars indicate the range of error of the determinations. The figure shows the lack of any close correlation between the spinal fluid sugar level and the sugar content of any isolated blood sample. The left hand figures indicate mg. per 100 cc.; the bottom row of figures is the case numbers.

The occurrence of low spinal fluid sugar in cerebral hemorrhage is not a new observation. Case 3869 was interesting because his febrile course and the high white cell count raised the question of infection of the meninges. Repeated cultures were negative, however; and he made an uneventful recovery. During the course of the rapid improvement, as the fluid became less bloody the white cells diminished and the sugar content rose to 0.53.

In Fig. 1 and Table II the sugar levels in blood and spinal fluid are presented in the order of increasing blood values. From the figure it is evident that no close dependence of spinal fluid sugar upon blood sugar is demonstrable. This is probably due to the fact that the sugar level of the blood is rapidly and constantly shifting, while cerebrospinal fluid is probably forming and absorbing only very slowly. The

TABLE II.

Sugar in Order of Increasing Blood Sugar.

Case No.	Blood plasma.	Spinal fluid.	Ratio, Spinal fluid/ Blood.	Case No.	Blood plasma.	Spinal fluid.	Ratio, Spinal fluid/ Blood.
51168*	74	60	0.81	3921	104	65	0.63
3849	83	60	0.71	3862	104	77	0.73
3921	87	69	0.79	3801	105	61	0.58
3827	90	53	0.59	3921	106	63	0.60
3985	90	66	0.74	3840	106	71	0.67
3842	91	61	0.67	2605	107	59	0.55
3898	91	74	0.81	3916	107	82	0.77
3827	91	64	0.71	3711	110	73	0.67
51164*	92	49	0.53	3821	110	65	0.60
3897	94	61	0.64	3873	111	71	0.64
3874	95	56	0.58	3917	113	73	0.64
3874	95	54	0.57	51021*	116	64	0.55
3921	97	55	0.56	3829	123	66	0.53
3815	100	79	0.79	3890	133	84	0.63
3789	101	73	0.72				
3853	102	70	0.68	3836	165	89	0.53
3805	102	65	0.64	3885	303	198	0.65
3874	103	69	0.67				

* Johns Hopkins Hospital Medical Service case numbers. All others are Henry Phipps Psychiatric Clinic numbers.

The last two cases are not included in the chart: No. 3836, because 2 hours before the lumbar puncture, an intravenous injection of 300 cc. of 5 per cent glucose had been given; No. 3885, because he was the only diabetic in the series.

sugar value of any one blood sample, therefore, may not represent the mean value from which the cerebrospinal fluid has been slowly elaborated and with which it is in equilibrium. However, wherever we had evidence that the blood sugar level had been maintained at a high point for some time, the fluid sugar was correspondingly raised, —as in Cases 3885 and 3836 (Table II, foot-note).

In our rather short and scattered series we find no correlations between the diseases encountered and either the absolute level of sugar in the cerebrospinal fluid or the ratio of fluid to blood.

2. The Chlorides in Venous Blood and Spinal Fluid.

There are, of course, more chlorides in the spinal fluid than in the blood. In only one instance, Case 3853, did we find a reversal of this relationship; and we had no opportunity to reexamine this patient and confirm our findings. This case, however, had the highest blood

TABLE III.

Sodium Chloride in Order of Increasing Blood Chlorides.

Case No.	Blood plasma.	Spinal fluid.	Ratio, Spinal fluid/ Blood.	Case No.	Blood plasma.	Spinal fluid.	Ratio, Spinal fluid/ Blood.
3869	514	652	1.27	3921	632	726	1.14
3869	530	650	1.22	3873	635	730	1.15
3921	560	727	1.30	3917	638	730	1.14
51021*	568	740	1.30	3827	645	743	1.15
51168*	585	717	1.23	3897	649	753	1.16
3885	600	747	1.24	3840	652	730	1.12
3921	602	753	1.25	3849	660	747	1.13
51164*	612	717	1.18	3711	662	790	1.19
3874	612	771	1.26	2605	664	769	1.15
3874	617	736	1.19	3985	674	764	1.13
3898	619	722	1.16	3829	729	771	1.06
3842	627	738	1.17	3853	767	730	0.95
3921	627	740	1.18				

* Johns Hopkins Hospital Medical Service case numbers. All others are Henry Phipps Psychiatric Clinic numbers.

chlorides encountered; and the fact that our data seem to indicate that the higher the blood chloride the lower is the ratio of spinal fluid chlorides to blood chlorides makes it at least possible that the findings are correct. A more plausible explanation of this case would be that the patient had had a sudden acute rise in his blood chloride content. From the experiments on dogs which are described below it is clear that such sudden changes in blood chemistry alter the fluid chemistry only slowly. It is then conceivable that the fluid had not yet had time to respond. In Table III and Fig. 2 the values

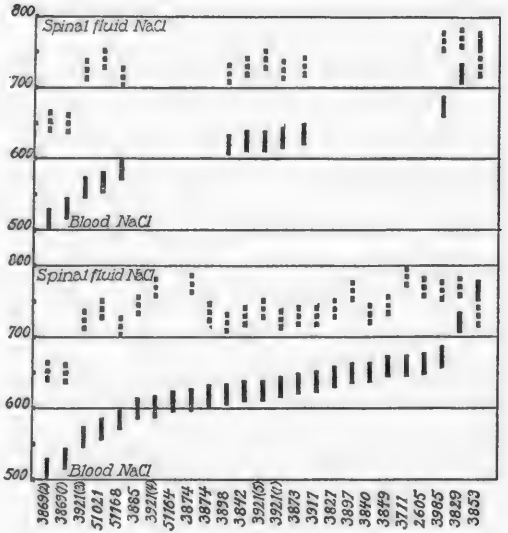

FIG. 2.

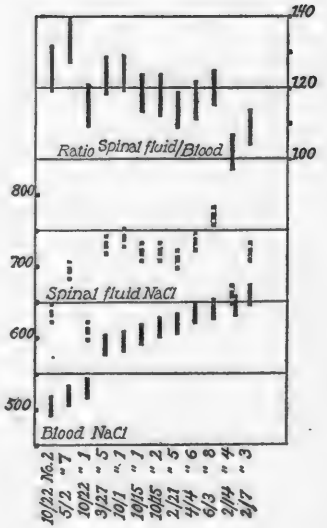

FIG. 3.

FIG. 2. The blood and spinal fluid chlorides from a series of patients, arranged in the order of increasing blood chlorides. The vertical bars indicate the range of error of the determinations. In the upper section of the figure three groups are picked out such that, allowing for the maximum error, no member of any group overlaps any member of the other groups. For the whole series, as well as for these three discrete groups, the falling ratio of spinal fluid chloride to blood chloride is quite evident. The left hand figures indicate mg. per 100 cc.; the bottom figures are the case numbers.

FIG. 3. The blood and spinal fluid chlorides from a series of dogs are presented, arranged as in Fig. 2. The values used are the averages of the first few determinations for each dog, before the intravenous injections have been given. The falling ratio is shown in the upper tier of symbols. The vertical bars again indicate the error of the determinations, and the resulting large error of the computed ratios. The figures on the left indicate mg. per 100 cc.; on the right are the ratios.

are arranged in the order of increasing blood chlorides; and the falling ratio is quite evident. The same thing is seen in the results of the analyses of repeated punctures on Case 3869, Case

TABLE IV.

Initial Chloride Values in Dogs, Arranged in Order of Increasing Blood Chlorides.

Dog No.	Date.	Anesthetic.	Interval.	Blood serum.	Spinal fluid.	Ratio, Spinal fluid/Blood.
			min.			
2	Oct. 22, 1924	Pantopon 4 mg. per kilo.	45	505	635	1.25
7	May 2, 1925	" 10 " " "	75	520	695	1.34
		Urethane ¾ gm. " "	45			
1	Oct. 22, 1924	Pantopon 4 mg. " "	45	530	610	1.18
5	Mar. 27, 1925			587	728	1.24
1	Oct. 1, 1924	Morphine ½ gr.	30	595	740	1.25
1	" 15, 1924	" ½ "	30	605	720	1.17
2	" 15, 1924	" ½ "	30	615	720	1.19
5	Feb. 21, 1925	Pantopon 15 mg. per kilo.	78	620	708	1.14
		Urethane ¾ gm. " "	33			
6	Apr. 4, 1925	Pantopon 10 mg. " "	75	632	735	1.15
		Urethane ¾ gm. " "	50			
8	June 3, 1925	Pantopon 10 mg. " "		640	770	1.20
		Urethane ¾ gm. " "				
4	Feb. 14, 1925	Pantopon 15 mg. " "	70	645	660	1.02
		Urethane ¾ gm. " "	25			
3	" 7, 1925	Pantopon 15 mg. " "	120	660	720	1.09
		Urethane ¾ gm. " "	60			

3921, and less perfectly on Case 3874. And, in a general way, this same falling ratio with rising blood chlorides is seen from the initial values obtained in the experiments on dogs (Table IV, Fig. 3).

From the work on patients, no explanation of this phenomenon is

apparent. The experimental work on animals, presented below, suggests that the Donnan membrane equilibrium may control the chloride distribution in this way.[1]

The actual ratios encountered ranged from 0.95 to 1.30, the majority falling between 1.10 and 1.25. No correlation between chloride ratios and disease processes was found.

3. The Non-Protein Nitrogen of the Cerebrospinal Fluid and of Venous Blood.

Except in one case, the non-protein nitrogen was lower in the cerebrospinal fluid than in the blood. This Case, No. 3869, was the one of cerebral hemorrhage which showed a very low sugar ratio. It is interesting that with improvement the sugar ratio rose, the non-protein nitrogen ratio fell, the chloride ratios fell slightly in response to a rising blood chloride level, and the white count in the fluid dropped. One is tempted to think, therefore, that the low sugar may have been due to a breaking down of sugar by an enzyme from the cells of the inflammatory reaction, and that the high non-protein nitrogen may have resulted from the proteolytic action of some enzyme on the proteins of the inflammatory exudate or of the hemorrhage itself.

In the other cases, the range of the ratio of spinal fluid to blood was from 0.51 to 0.85 for whole blood, and from 0.65 to 0.92 for plasma. With these substances, as with sugars, the fluid level seems not to depend on blood level very closely, although large and prolonged changes are seen to result in corresponding changes in fluid values, —as in Case 51021 in uremic coma, and Case 3836, a nephritic.

In contrast to the findings with the chlorides, there seems to be here a slight tendency for the ratio of spinal fluid to blood to rise as the level of non-protein nitrogen in the blood rises; but this is not as clear-cut as is the reverse tendency on the part of the chlorides.

The data are presented in Table V.

[1] Since sending this to press, it has been our privilege to see the data which have been collected by Dr. F. Fremont-Smith at the Massachusetts General Hospital, Boston, Mass., and which are soon to be published. Determining, among other things, the plasma proteins, and blood and fluid chlorides, on a much larger and more heterogeneous group of patients, he has reached results which in all essentials correlate with and amplify our own.

4. The Interrelation of Chlorides, Sugar, and Non-Protein Nitrogen.

In Table VI, the chloride, sugar, and non-protein nitrogen ratios are arranged together in the order of their increasing blood chloride values. This order was chosen because the correlated decrease of the chloride ratios brought at least one of these series into a fairly orderly

TABLE V.

Non-Protein Nitrogen in Order of Increasing Blood Values.

Case No.	Whole blood.	Spinal fluid.	Ratio, Spinal fluid/Blood.
3885	33	25	0.75
3842	33	23	0.69
3921	37	18	0.51
3711	39	31	0.82
51021*	199	170	0.85
Case No.	**Blood plasma.**	**Spinal fluid.**	**Ratio, Spinal fluid/Blood.**
3921	18	13	0.65
51164*	22	17	0.79
3921	22	15	0.68
3849	23	15	0.65
3869	25	17	0.69
3869	25	29	1.13
3921	26	17	0.65
3874	28	19	0.67
51168*	30	18	0.59
3874	32	21	0.65
3711	32	31	0.93
3869	34	39	1.15
3873	40	31	0.78
3836†	182	167	0.92

* Johns Hopkins Hospital Medical Service case numbers. All others are Henry Phipps Psychiatric Clinic numbers.

† 2 hours after intravenous glucose.

sequence. For the whole group of heterogeneous cases arranged in this way, it is not possible to detect any definite interrelationships between the three constituents. The error of the ratio computation is, of course, large and may be sufficient to mask any relationship that might exist. But as far as one can see, there is no general motion of all the ratios in one direction, nor any definite tendency to compen-

sate for one another by moving in opposite directions. The ratios when added together, however, show less divergence than any indi-

TABLE VI.

Combined Ratios Spinal Fluid/Blood Arranged in Order of Increasing Blood Chlorides.

Case No.	Blood chloride.	Chloride ratio.	Sugar ratio.	N-P N ratio.	Total ratios.
3869* (2)	514	1.27	0.33	1.15	2.75
3869* (1)	530	1.22	0.38	1.13	2.73
3921 (2)	560	1.30	0.79	0.65	2.74
51021†	568	1.30	0.55	0.85‡	2.70
3874 (3)	584	1.24	0.58	0.65	2.47
51168†	585	1.23	0.81	0.59	2.63
3869 (3)	587	1.18	0.53	0.69	2.40
3885	600	1.26	0.65	0.75‡	2.66
3921 (3)	602	1.25	0.60	0.69	2.54
51164†	612	1.18	0.53	0.79	2.50
3874 (2)	612	1.26	0.57		
3874 (4)	617	1.19	0.67	0.67	2.53
3898	619	1.16	0.81		
3842	627	1.17	0.67	0.69‡	2.53
3921 (4)	627	1.18	0.63	0.68	2.49
3921 (1)	632	1.14	0.56	0.51‡	2.21
3875	635	1.15	0.64	0.78	2.57
3917	638	1.14	0.64		
3827 (2)	645	1.15	0.71		
3897	649	1.16	0.64	0.75	2.55
3840	652	1.12	0.67		
3849	660	1.13	0.72	0.65	2.50
3711	662	1.19	0.67	0.93‡	2.79
2605	664	1.16	0.55		
3985	674	1.13	0.74		
3829	729	1.06	0.53		
3853	767	0.95	0.68		
3836*	812	1.15	0.53	0.92	2.60

*Special cases, separated from the rest of the chart for special reasons. *Cf.* Table I.

†Johns Hopkins Hospital Medical Service case numbers. All others are Henry Phipps Psychiatric Clinic numbers.

‡Ratios calculated from spinal fluid over whole blood. All others are spinal fluid over plasma.

vidual series,—suggesting more of the latter process than of the former.

If, however, one takes the ratios for a single individual who was punctured four times, one finds in this one instance a tendency for all of the ratios to move together, as though there were a concentration or dilution of the fluid as a whole with respect to all of its constituents.

Case 3921.

Cl	Sugar.	N-P N.
1.14	0.56	0.51
1.18	0.63	0.68
1.25	0.60	0.69
1.30	0.79	0.65

II. The Experimental Work on Dogs.

Small doses of morphine or pantopon enabled one to make a single cisterna puncture, but for the longer experiments described below it was necessary to secure a more profound anesthesia. We used 10 to 15 mg. of pantopon per kilo, subcutaneously; followed in half an hour by ¾ gm. of urethane per kilo, by stomach tube. It is evident from our figures that this caused high hemoglobin and high sugar values; but to give us information as to the direction in which these changes were taking place, we took repeated blood samples at about 15 minute intervals before securing our first cerebrospinal fluid sample.

The dogs lay on their right sides facing a large hot box. In this box stood a 2½ liter graduated Mariotte bottle holding the fluid which was to be injected intravenously. The rubber tubing from the bottom outlet of the Mariotte bottle was led through a du Noüy pump (5), only about 12 inches of the tubing projecting outside of the warm box, to reach the animal's leg. In this way the fluid was kept at, or near, body temperature. The fluid, the bottle, and the tubing were sterilized the night before the experiment.

As can be seen from the tables and curves, our procedure was to secure repeated samples of left heart blood, then to perform a cisterna puncture and collect in graduated tubes approximately equal samples of spinal fluid as long as it would run freely and spontaneously. The size of the samples depended on the number of determinations to be made. After collecting the first rush of fluid we usually took one or two very slowly appearing samples before starting the injection. Throughout the experiment, when the flow was not too rapid, we secured a sample of left heart blood at the end of each spinal fluid collection. Thus we secured, not the initial base line from which the blood picture was altered by the anesthesia and with which the spinal fluid was in equilibrium before the experiment was undertaken, but a fairly complete picture of the alterations in the blood and in

the fluid from that point on. When the outflow of spinal fluid fell to a rate of a drop or two per minute, a new sample of left heart blood was taken, and at once an intravenous injection of a hypotonic solution was begun. This renewed the outflow of fluid, and samples were collected throughout the injection period, with left heart punctures at the end of each fluid collection as before. After the termination of the injection as many samples as possible were collected during the recovery period.

Aseptic precautions were observed in all of these experiments and no infections occurred. 2 days after an experiment the dogs were in excellent shape, and after an interval of a few weeks were used again for these and other similar procedures.

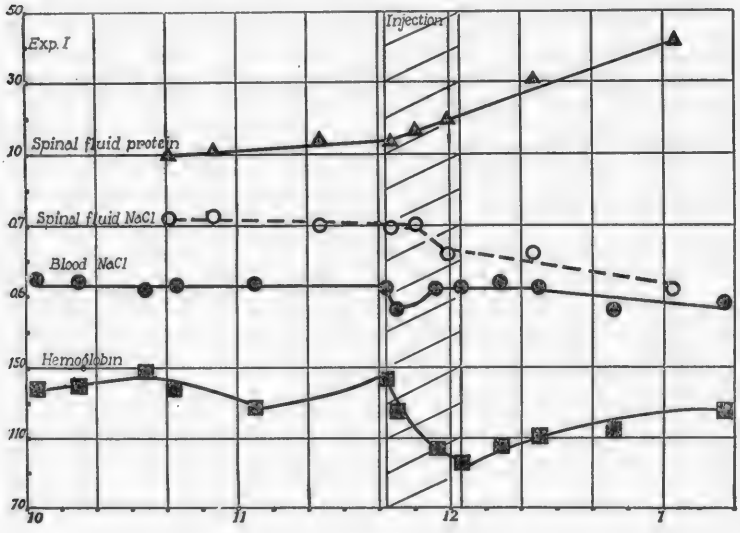

FIG. 4.

FIGS. 4 to 9. The hours are numbered on the abscissæ, each 10 minute interval being indicated by the small check, and each vertical line representing 20 minutes. On the ordinates, the hemoglobins are indicated in per cent (Haldane), serum proteins in gm. per 100 cc., spinal fluid proteins in mg. per 100 cc., chlorides in gm. or mg. per 100 cc., and sugars in mg. per 100 cc.

In each case the shaded area indicates the duration of the injection.

All spinal fluid values are plotted in the middle of the collection period. The interval between the spinal fluid symbols indicates roughly, therefore, the rate at which the sample has appeared; and the blood value that just precedes and that just follows any spinal fluid symbol marks almost exactly the beginning and end of the collection.

Two dogs died from puncture of a coronary artery. The rest gained weight in the months during which they were used in these and other closely allied experiments.

All blood samples were drawn into syringes containing oil; and were then discharged into centrifuge tubes under oil. A sample was taken at once for hemoglobins and then the oil was pipetted off until only a thin film remained over the blood, and over this a layer of low melting paraffin (42°C.) was poured. The samples were then allowed to stand until it was convenient to centrifuge them—usually about an hour after the collection.

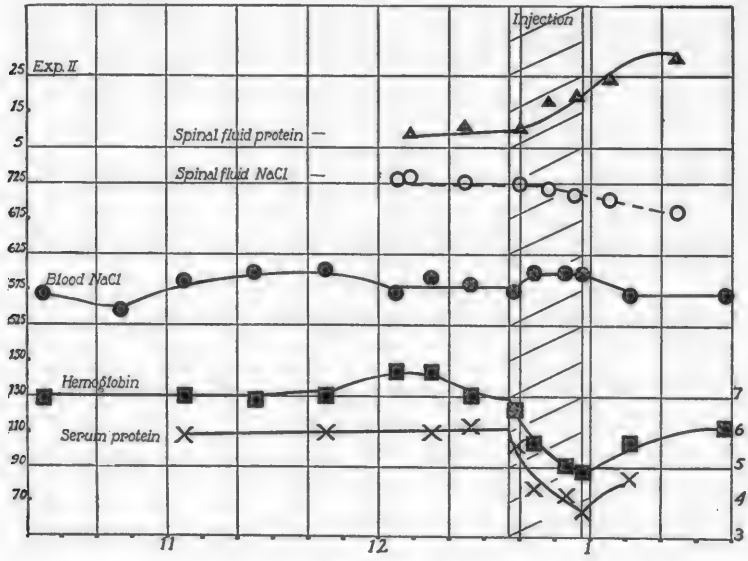

FIG. 5.

With few exceptions all chemical examinations were made on the day of the experiment. Hemoglobins were read against the Newcomer glass standard (4). Serum proteins were determined by doing Kjeldahl determinations of total nitrogen, Folin determinations of the non-protein nitrogen (3), subtracting the latter from the former and calculating the proteins. Chlorides were determined by the Whitehorn method (7), in the first two experiments, and by the Van Slyke (6) method in the rest. Sugars were determined by the Folin and Wu method (2).

In all of the figures, the size of the symbols indicates closely the error of the method. In Figs. 4 to 9, and in the corresponding Tables

VII to XII, the data of six of our nine experiments on dogs are presented. In the first two experiments hypotonic saline solutions were injected; in the rest, hypotonic glucose. No hemolysis occurred in any of the experiments presented here. Because it is extremely

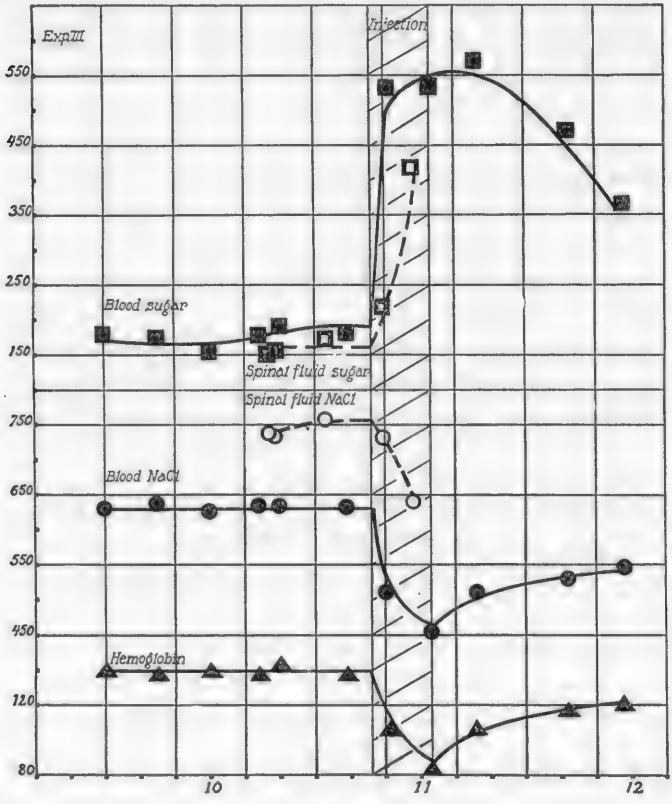

FIG. 6.

difficult to prevent hemolysis of dog's blood in the presence of oxalate, serum was used instead of plasma in all of these six experiments.

We are unable to make any assumptions at this time as to the relations of the normal fluid to the fluid formed under the influence of these

injections. We do not know at present whether the latter is a choroid plexus product, a transudation through the small vessels of

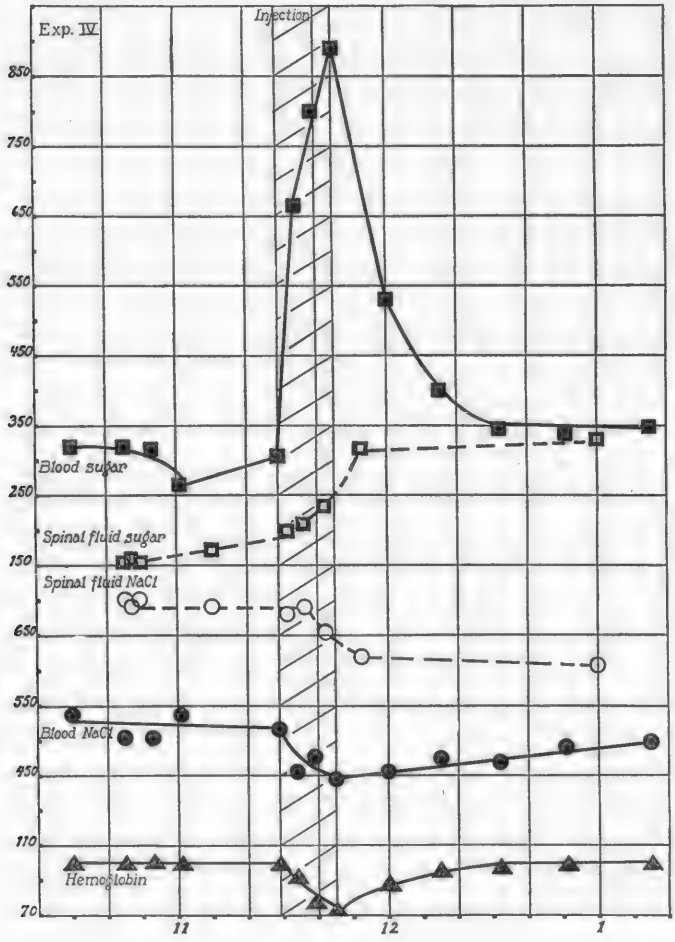

FIG. 7.

the parenchyma, or both, although this is an important question for the interpretation of these results.

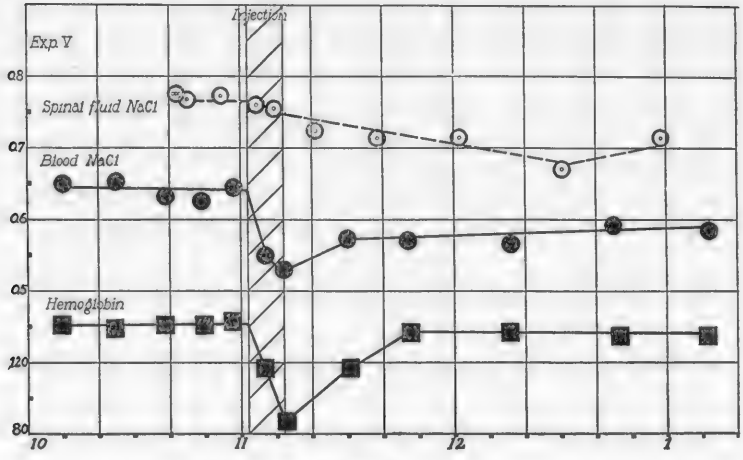
Injection
Exp. V
Spinal fluid NaCl
Blood NaCl
Hemoglobin
0.8
0.7
0.6
0.5
120
80
10
11
12
1

FIG. 8.

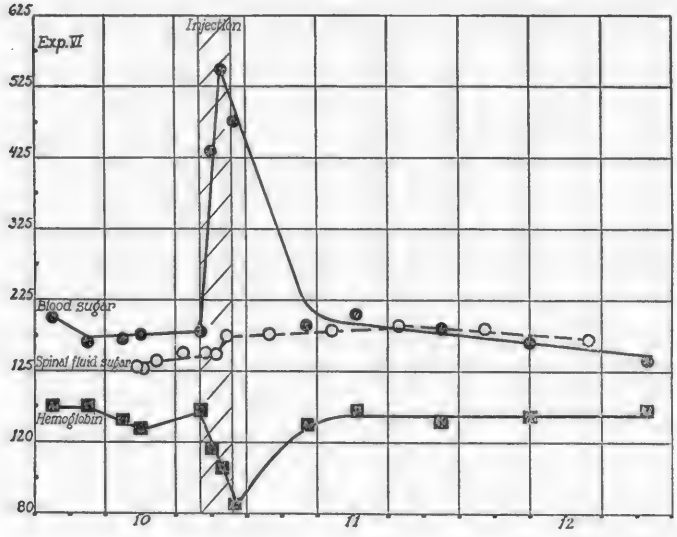
Injection
Exp. VI
625
525
425
325
225
125
120
80
Blood sugar
Spinal fluid sugar
Hemoglobin
10
11
12

FIG. 9.

Summary of the Experimental Data.

1. Experiments I and II (Tables VII and VIII, Figs. 4 and 5) show that:

(*a*) Slight hemoglobin variations can occur without corresponding variations in the serum proteins; but during the intravenous infusion the hemoglobin gives an adequate rough measure of the degree of

TABLE VII.

Experiment I.—February 21, 1925. Dog 5; weight 17.5 kilos.
Anesthesia: Pantopon, subcutaneously, 15 mg. per kilo—255 mg. at 8.45 a.m. Followed by slight purging.
Urethane, by stomach tube, 0.75 gm. per kilo—12.75 gm. at 9.30 a.m.

Time.	Blood No.	Spinal fluid No. (2.5 cc. samples).	Injection.	Hemoglobin (Haldane).	Spinal fluid protein.	Serum NaCl.	Spinal fluid NaCl.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
10.03	1			138		624	
10.15	2			141		621	
10.35	3			148		610	
10.43–10.45		1			10.5		711
10.45	4			138		617	
10.45–11.05		2			11.5		713
11.05	5			128		618	
11.05–11.42		3			14.4		700
11.42	6		Injection 0.5 per cent NaCl begun.	144		612	
11.42–11.45		4			13.0		698
11.45	7		350 cc.	125		582	
11.45–11.56		5			17.1		702
11.56	8		900 "	104		610	
11.56–12.03		6			19.6		659
12.03	9		Injection stopped: 1300 cc.	96		612	
12.14	10			106		619	
12.25	11			111		612	
12.03–12.46		7			31.0		662
12.46	12			115		582	
12.46–1.23		8			—		607
1.23	13			125		590	

TABLE VIII.

Experiment II.—March 27, 1925. Dog 5; weight 17.3 kilos.

Anesthesia: Pantopon subcutaneously, 10 mg. per kilo—173 mg. at 9.00 a.m. Followed by slight vomiting.

Urethane, by stomach tube, 0.75 gm. per kilo—13 gm. at 9.35 a.m.

On table at 10.00 a.m.

Time.	Blood No.	Spinal fluid No. (about 1.5 cc. samples).	Injection.	Hemoglobin.	Serum protein.	Spinal fluid protein.	Serum NaCl.	Spinal fluid NaCl.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
10.24	1			128	—		558	
10.47	2			—	—		546	
11.05	3			130	5.87		587	
11.25	4			128			599	
11.45	5			130	6.01		604	
12.05–12.06		1				15.8		728
12.06	6			144			571	
12.06–12.12		2				9.3		734
12.14	7			144	6.04		591	
12.12–12.26		3				10.7		727
12.26	8			130	6.14		582	
12.37			Injection 0.5 per cent NaCl begun: 3.7 cc. per kilo per min.					
12.37–12.43		4				10.5		725
12.39	9			123	5.63		571	
12.44	10			105	4.38		601	
12.43–12.53		5				18.2		718
12.53	11			91	4.17		601	
12.53–12.57		6				19.5		711
12.57	12		1300 cc. Injection stopped.	88	3.71		599	
12.57–1.11		7				24.6		704
1.11	13			105	4.76		569	
1.11–1.48		8				23.6		687
1.48	14			113			569	

dilution of the blood stream with respect to its protein content. (This is further shown in two of the experiments not presented here.)

(*b*) The injection of 1300 cc. of a 0.5 per cent saline solution into a

TABLE IX.

Experiment III.—April 4, 1925. Dog 6; weight 18 kilos.

Anesthesia: Pantopon subcutaneously, 10 mg. per kilo —18 mg. at 8.25 a.m. Followed by purging.

Urethane, by stomach tube, 0.75 gm. per kilo —13.5 gm. at 8.40 a.m.

On table 9.00 a.m.

Time.	Blood No.	Spinal fluid.	Injection.	Hemoglobin.	Spinal fluid protein.	Serum NaCl.	Spinal fluid NaCl.	Serum sugar.	Spinal fluid sugar.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
9.30	1			141		632		171	
9.45	2			138		638		167	
10.00	3			141		627		152	
10.14	4			138		635		177	
10.16–10.17		1(3cc.)			11.2		738		150
10.17–10.20		2(3 ")			11.2		735		154
10.20	5			143		635		200	
10.39	6			138		633		182	
10.20–10.46		3(3 ")			75.2		754		174
10.46			Injection 3 per cent glucose begun.						
10.46–10.53		4(3 ")			171.4		727		220
10.51	7			108		513		533	
10.53–11.03		5(2 ")					641		420
11.03			Injection 1550 cc. stopped.						
11.04	8			80		455		533	
11.16	9			108		512		571	
11.42	10			119		530		471	
11.58	11			121		548		364	

An accidental dislocation of the needle caused a little blood to appear in the 5th spinal fluid sample. Despite centrifuging, its sugar values are therefore a little high and its chlorides a little too low. Because of the blood, no later samples of fluid were taken.

17 kilo dog in 20 minutes (a rate of about 3.7 cc. per kilo per minute) caused little reduction in the chloride level of the dog's serum, despite the fact that the original serum chlorides were approximately 0.6 per cent. In Experiment I there is a sharp initial drop of 30 mg., 3

TABLE X.

Experiment IV.—May 2, 1925. Dog 7; weight 16 kilos.
Anesthesia: Pantopon subcutaneously, 10 mg. per kilo—160 mg. at 9.15 a.m.
Urethane, by stomach tube, 0.75 gm. per kilo,—12 gm. at 9.45 a.m.

Time.	Blood No.	Spinal fluid.	Injection 3 per cent glucose.	Hemoglobin.	Spinal fluid protein.	Serum NaCl.	Spinal fluid NaCl.	Serum sugar.	Spinal fluid sugar.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
10.30	1			101		538		320	
10.45	2			101		503		320	
10.45–10.46		1(2.7cc.)			10.0		702		154
10.46–10.48		2(2.5 ")			8.8		690		160
10.48–10.51		3(3.2 ")			11.2		702		154
10.51–11.29		4(3.2 ")			14.2		690		173
10.53	3			103		503		319	
11.01	4			100		538		266	
11.29	5		Injection 3 per cent glucose begun.	101		515		307	
11.29–11.34		5(3.2 ")			19.9		679		200
11.34	6			93		456		666	
11.35			500 cc.						
11.34–11.39		6(3.1 ")			33.4		690		210
11.39	7		800 "	78		468		800	
11.39–11.45		7(3.2 ")			98.4		655		235
11.45	8		1200 " Injection stopped.	74		445		888	
11.45–12.00		8(2.6 ")			240.0		620		317
12.00	9			90		456		532	
12.00–1.15		9(2.4 ")			476.0		608		334
12.15	10			96		474		400	
12.32	11			99		468		347	
12.51	12			100		491		339	
1.15	13			100		497		347	

minutes after the injection began—a drop that is just at the extreme limits of error of the method. 11 minutes later, with the injection still in progress, the chlorides have returned to their initial level.

Two factors probably account for this. In the first place, in the blood stream which is diluted by an intravenous injection, each sample

TABLE XI.

Experiment V.—June 3, 1925. Dog 8; weight 14 kilos.
Anesthesia: Pantopon subcutaneously, 10 mg. per kilo—140 mg. at 9.20 a.m.
Followed by purging and vomiting.
Urethane, by stomach tube, 0.75 gm. per kilo—10.5 gm. at 9.45 a.m.
On table at 10.00 a.m.

Time.	Blood No.	Spinal fluid No.	Injection.	Hemoglobin (Haldane).	Serum NaCl.	Spinal fluid NaCl.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*
10.10	1			140	649	
10.25	2			138	653	
10.39	3			140	632	
10.41 –10.41½		1 (1.3cc.)				775
10.41½–10.42		2 (1.4 “)				775
10.42 –10.43		3 (1.4 “)				774
10.43 –10.47		4 (1.2 “)				767
10.47 –11.02		5 (1.7 “)				772
10.49	4			140	626	
10.58	5			143	645	
11.02			Injection started, 3 per cent glucose.			
11.02 –11.07		6 (1.1 “)				760
11.07	6		400 cc.	117	550	
11.07–11.12		7 (1.3 “)				755
11.12	7		800 “ Injection stopped.	87	530	
11.12 –11.30		8 (1.1 “)				725
11.30	8			117	573	
11.30 –11.47		9 (0.55 “)				715
11.47	9			138	570	
11.47 –12.16		10 (0.55 “)				715
12.16	10			138	566	
12.16 –12.45		11 (0.7 “)				670
12.45	11			135	593	
12.45 –1.12		12 (0.6 “)				715
1.12	12			135	585	

of blood taken for analysis will consist of more chloride-containing water and less chloride-free protein; so that a slight drop in the chlorides per cc. of the aqueous component of the blood stream can be

TABLE XII.

Experiment VI.—June 4, 1925. Dog 5; weight 21 kilos.

Anesthesia: Pantopon subcutaneously, 10 mg. per kilo—210 mg. at 8.30 a.m. Followed by purging and vomiting.

Urethane, by stomach tube, 0.75 gm. per kilo—16.5 gm. at 9.00 a.m.

On table 9.20 a.m.

Time.	Blood No.	Spinal fluid No. (1.2 cc. each).	Injection.	Hemoglobin.	Serum sugar.	Spinal fluid sugar.
				per cent	*mg. per 100 cc.*	*mg. per 100 cc.*
9.35	1			140	210	
9.45	2			140	167	
9.55	3			132	171	
9.57		1				133
		2				133
to		3				125
10.00		4				125
10.00	4			127	176	
10.00–10.02		5				125
10.02–10.07		6				136
10.07–10.17		7				152
10.17	5		Injection 2.8 per cent glucose begun.	138	181	
10.17–10.20		8				153
10.20	6		250 cc.	117	439	
10.20–10.23		9				150
10.23	7		475 "	106	548	
10.23–10.26		10				176
10.26	8		600 " Injection stopped.	85	476	
10.26–10.47		11				178
10.47	9			130	190	
10.47–11.01		12				183
11.01	10			138	207	
11.01–11.25		13				190
11.25	11			132	184	
11.26–11.50		14				184
11.50	12			135	167	
11.50–12.23		15				171
12.23	13			138	142	

masked by the fact that one is analyzing more water in each cc. of blood examined. In the second place, on the basis of the Donnan

membrane equilibrium, the lowered protein content of the blood stream during the injection should lessen the tendency of the blood to transfer its negative ions to those body fluids which are lower in proteins than itself. Thus, when a 0.5 per cent saline solution is injected into a blood stream which is 0.6 per cent saline, the protein dilution may cause a return of Cl ion from body fluids to blood stream, and may prevent in this way a lowering of the chloride content of the blood.

(*c*) Since, in these two experiments, the chloride content of the blood stream was not perceptibly lowered for more than a few moments, it must be this protein dilution which accounts for the lowering of the spinal fluid chlorides in the samples collected after the termination of the injection. It seems to us justifiable to assume that these samples were formed under the influence of the injection, and that the lag in the manifestation of this effect represents merely the time which it takes for the newly formed fluid to appear at the needle. We conclude, therefore, that the fluid formed from a blood stream with relatively constant chloride content but a lowered protein content, received from the blood less chloride.

(*d*) In these and all of the other experiments, there was no tendency for the proteins of the fluid to follow those of the blood. On the contrary they tended to rise sharply during and after the injections. This increase in the proteins of the cerebrospinal fluid, in so far as it could have any effect at all, would accentuate the results of the reduction of the proteins of the blood stream.

2. In Experiments III and IV (Tables IX and X, Figs. 6 and 7), it is shown that a far sharper reduction in the spinal fluid chlorides can be produced by injecting a hypotonic glucose solution; thus diminishing both the chloride concentration and the proteins of the blood stream. In these two experiments, relatively large samples of fluid had to be collected, however, and the exact shape of the curves could not be followed. From Experiment IV it would appear as if a falling chloride level in the fluid continued to appear after the injection was over, even against an increasing blood chloride concentration. In Experiment V (Table XI, Fig. 8), however, the analysis of smaller fluid samples shows that, allowing for the delay in the appearance of the fluid at the needle, the fluid chlorides follow closely the blood

chlorides under the experimental conditions. And, doubtless, a drop-by-drop analysis by micro methods would show an even closer relationship.

3. The determinations of reducing bodies, after the injections of glucose solutions in Experiments III, IV, and VI (Table XII, Fig. 9), show that the sugar level in the fluid tends to follow that of the blood stream. It is made evident, however, even more clearly than in the chloride studies that the time factor is important. When the high sugar level of the blood stream is maintained for several minutes, the fluid values, after a short lag, climb high. But where, as in Experiment VI, the peak is very brief, the rise in the fluid values is by no means comparable. Here again, however, micro methods applied to drop-by-drop samples are needed to fill in the details of the correlation.

4. In Experiments III and IV the rise in protein content of the fluid after glucose injections was phenomenal—in one, 171 mg., and in another, 476 mg. appearing. No explanation of this is apparent.

5. It is commonly assumed that if the initial rush of fluid is taken in successive fractions, the first fractions represent fluid from the cord, brain stem, brain base, etc., whereas the later fractions are largely ventricular fluid. However this may be, those experiments in which we divided this initial flow of fluid into small samples gave us results which suggest that:

(*a*) There is no difference between the chloride content of the various samples.

(*b*) There is a fairly constant tendency for the sugar content to increase as the flow of fluid slows up.

(*c*) The protein content of the first sample is usually just recognizably higher than that of the second,—but the protein, like the sugar content, rises again as the flow slows down.

CONCLUSIONS.

1. If due allowance is made for the slow rate of elaboration of cerebrospinal fluid and for the time that it takes for newly formed fluid to emerge from a needle in the cisterna, the amount of sugar, chloride, and non-protein nitrogen in the fluid seems to follow in a general way their concentrations in the blood stream.

2. It is equally evident, however, that for any substance other factors besides its concentration in the blood stream play a part in its distribution between blood and cerebrospinal fluid.

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